

**The emission of volatile organic compounds (VOCs) from rotting fruits and
wilting flowers**

Charlene Ragubeer

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SUMMARY

Plants and the microbes that feed on them produce a high diversity of volatile organic compounds (VOCs) that often mediate interspecies communication with other organisms. Two main functions of VOCs emitted by plants are to attract pollinating animals to flowers and seed dispersers to fruits; while volatiles emitted by microbes that feed on plant material can be used as cues by animals searching for food and can also facilitate dispersal of the microbes.

The emission of VOCs from flowers and fruits often shows temporal changes that are characteristic for the different ripening stages of the plant organs. In many plants the VOC emission not only increases but also changes in its chemical composition during flower opening and fruit ripening respectively. In addition, VOC emission into the atmosphere carries information about the physiological status and stresses of the plant. However, the vast majority of studies that deal with the function of VOCs, e.g. for attracting pollinating animals or seed dispersers, focus only on those components that are emitted during the time when the plant shows the highest attractiveness for interaction partners. It is reasonable to believe, however, that the decisions made by animals, in terms of host preference and selection, are not only based on the chemical components that are emitted during times of optimal (nutritional) condition. The decision to utilize a flower/fruit is most likely also based on components that indicate to animals that a food source is not worth using, unpalatable, or toxic. For example, early stage flowers and unripe fruits have a low nutritional value, and late stage (wilted) flowers and rotten fruits may, in addition to already depleted resources, also contain toxic chemicals from microbial decomposition of the plant tissue.

Compounds that are emitted from flowers and fruits are often difficult to detect with conventional headspace extraction techniques that use solvents. This is a particular problem for researchers interested in fermentation volatiles because many of the emitted compounds (e.g. ethyl acetate, acetic acid, acetoin) overlap in their retention time with solvents that are commonly used for extracting these compounds such as hexane, acetone, or dichloromethane. In this thesis two commonly used extraction techniques were compared: (i) solid phase micro-extraction (SPME) and (ii) micro solid phase extraction (Micro SPE), both the techniques are used to collect fermentation and floral VOCs. For Micro SPE two different chromatography columns (DB5 and Carbowax) were used to determine which is more sensitive in identifying compounds.

For this, a floral and fermentation standard mixture was created by using compounds that represent sweet smelling flowers and rotting/fermenting fruits. Significant differences in the absolute emission of compounds, when using SPME and Micro SPE were found. There were also significant differences with the use of a DB5 and Carbowax column. The selection of the appropriate extraction technique for collection of VOCs should be based upon the type of application and availability of the necessary equipment. From this study, I found that Micro SPE worked better for collecting early to late stage VOCs of flowers and fruits, particularly if samples need to be collected under field conditions.

To characterise the typical fermentation volatiles from flowers, the temporal variation in the emission of floral VOCs from the freshly opened to the wilted stages of three plant species namely: *Hymenocallis littoralis*, *Dendrobium chrysotoxum* and *Camellia reticulata* were investigated. The study revealed that there were significant differences in the absolute amounts and in some species also in the number of compounds emitted between early stage flowers and wilted flowers. *Hymenocallis littoralis* had a higher absolute emission of compounds on day one of flowering and thereafter emission decreased. However, no differences in the number of compounds were detected for this species. The VOCs of *H. littoralis* on the first day of sampling included: linalool, (1*Z*)-2-methylbutanal oxime, and 2-methyl-6-methylene-1,7-octadien-3-one, however the composition during the wilted stage included: (3*E*)-3-hexenyl acetate, heptenal, nonanal, and 2,6-dimethyl-7-octen-2-ol. There was a difference in the absolute emission of compounds for *D. chrysotoxum*, the emission increased until day six and thereafter decreased. There was also a difference in the number of compounds emitted, with more compounds being emitted from day 1 to 9 of sampling. The VOCs that contributed to the overall scent of the flowers of *D. chrysotoxum* from day 1 to 11 included: myrcene, linalool, limonene, 3,7-dimethyl-1,3,7-octatriene and α -pinene, however as the flowers began to wilt from day 12 to 13, the VOC composition changed and 4-dimethyl-1-heptene, limonene, terpinen-4-ol and (Z)-verbenol contributed to the late stage scent.

For *Camellia reticulata* significant changes in the absolute emission and the number of compounds during the fresh and wilted flowering stages were detected. It was found that more compounds were emitted during the wilted stage. During the early stage of flowering, ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate, linalool, and 2H-pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl- contributed to the scent of the flowers. During the wilted

stage, benzaldehyde, benzyl alcohol, (*E*)-2-Hexen-1-ol, and 2-Heptanol contributed to the scent of the flowers.

To investigate typical fermentation volatiles three plant species, *Musa acuminata* (banana), *Pyrus communis* (pear) and *Rothmannia globosa*, were selected. For the three selected species, the temporal variation in the emission of VOCs from fruits during the ripening process (i.e. ripe to overripe stages) were investigated. Significant differences in the absolute amount and number of compounds emitted were detected. For *M. acuminata*, there was a higher absolute emission and number of compounds emitted from sampling day 1 to 10. Acetoin and 2,3-butanediol contributed to the scent of rotting *M. acuminata*. For *P. communis*, more absolute emission took place on day 1 of sampling and thereafter decreased, followed by an increase at day 35 of sampling and thereafter decreasing. Hexyl acetate, n-butyl butanoate, 1-hexanol and n-amyl acetate were found during the ripe stage of the fruit, and the VOC composition changed during rotting with the occurrence of ethyl acetate, acetic acid, 3 methyl-1-butanol, phenylethyl alcohol and benzaldehyde.

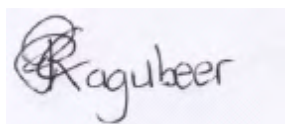
Rothmannia globosa was sampled during the ripe and overripe stages. Higher absolute emission and number of compounds were emitted during the ripe stage of the fruit. 1-hexanol contributed to the scent of *R. globosa* during the ripe stages, however during the rotting stage, n-butyl acrylate, benzaldehyde, 1-butanol and 2-ethyl-1-hexyl acetate were found in samples.

The findings of this study have relevance for researchers that are interested in the role of temporal VOC changes for the behaviour of pollinators and seed dispersers. Furthermore, it may be beneficial to researchers interested in the chemicals that are emitted by flowers that mimic rotting fruit volatiles. It is likely that microbial VOCs together with VOC changes initiated by the plant play an ecological role for host selection in animals. However, there is still a gap in our knowledge regarding the functional role of these VOCs and further studies are needed to investigate how such changes affect animal behavior and host selection. In addition, some of the findings of this study might be of interest for more applied areas such as horticulture and agriculture where the detection of microbial VOC signatures is important for detecting microbial pathogens, early senescence, and decomposition of plant tissue.

PREFACE

All the work described in this thesis was carried out at the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, from January 2013 to December 2015, under the Supervision of Dr. Andreas Jürgens and Co-supervision of Prof. Steven Johnson.

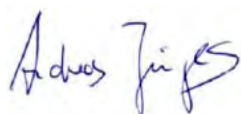
This thesis, submitted for the degree of Master of Science in the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, represent original work by the author and has not been submitted in any form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.



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Charlene Ragubeer

December 2015

I certify that the above statement is correct and as the candidate's supervisor, I have approved this thesis for submission.



.....
Doctor Andreas Jürgens

Supervisor



.....
Professor Steven Johnson

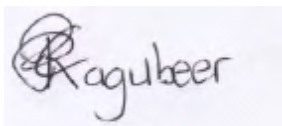
Co-supervisor

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DECLARATION – PLAGIARISM

I, Charlene Ragubeer, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Charlene Ragubeer

December 2015

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Chapter 1

The role of microbial volatiles as olfactory signals for pollinators and seed-dispersers: A literature review

Abstract

The life cycle of angiosperms is characterized by two events, pollination and seed dispersal, where animals are utilized for the transport of pollen and seeds. During these life stages flowers and fruits may produce volatile organic compounds (VOCs) to signal the presence of a potential reward, e.g. floral nectar or carbohydrate rich fruit tissue, to animals. It has been shown in many studies that VOCs play an important role in mediating between the self-interest of plants to attract animal vectors for pollen and seed dispersal and the self-interest of animals to find food (or any other reward flowers and fruit may offer). However, trophic interactions of flowers and fruits with microorganisms may interfere with plant-animal signalling because they produce characteristic VOCs that can potentially affect the behaviour of pollinators and seed dispersers and in consequence the reproductive success of plants. Signals produced by microorganisms may indicate the presence of food thereby attracting animals, whereas signals that indicate the absence of food may repel animals. Pollinators may use the emission of late stage VOCs as a cue to avoid old flowers of which resources have been depleted. In this review I investigate decomposition- and microbial VOCs emitted from flowers and fruits and their role in mutualistic interactions of angiosperms. Improving our knowledge on microbial VOCs and how they affect animal responses to flowers and fruits is crucial for understanding the ecology and evolution of interactions between flowering plants and their mutualistic animal partners.

Keywords: Pollination, seed dispersal, volatiles, fruits, flowers, yeast, bacteria, wilting, senescence.

1.1. Introduction

The sessile lifestyle of flowering plants makes it difficult to find mating partners or to disperse to new habitats. To overcome this problem angiosperms have evolved mechanisms to exploit the mobility of animal partners by utilizing them as transporters of pollen and seeds. The transport service of animal interaction partners is often facilitated by attracting them to food sources that flowers (e.g. nectar) and fruits (e.g. fleshy fruit tissue) provide (Fleming and Kress, 2011). To advertise potential food sources to pollinators and seed dispersing, animals and plants produce olfactory and visual signals. The important role of volatile organic compounds (VOCs) as olfactory cues for attracting mutualistic animals has been shown in many studies (Baldwin, 2010, Das et al., 2013). Floral- and fruit- VOCs (often together with colour signals) may convey information to pollinators and fruit/seed dispersers not only on the presence but also on the quality, quantity and abundance of potential rewards (Farre-Armengol et al., 2013).

The typical life cycle of successfully pollinated flowers can be divided into different phases of plant life-history (**Figure 1**). All these phases may be associated with characteristic VOCs emitted from floral tissue (e.g. petals, bracts) and fruit tissue (e.g. receptacle) attached to the reproductive parts (ovary/seeds) of the plant. It is generally assumed that the VOCs produced during the flowering and fruiting stages have evolved to attract specific mutualistic interaction partners (Junker and Bluthgen, 2010, Knudsen et al., 2006, Rodriguez et al., 2013). Furthermore, flowers and fruits associated with similar interaction partners show convergence regarding their VOC composition. This indicates that the different VOC patterns found in floral bouquets and also in fruit aromas are partly the result of a selection process that is mediated by animals with different sensory capabilities and preferences (Knudsen et al., 2006).

However, visual and olfactory signals of flowers and fruits are not necessarily honest signals to animals and there are many examples of deceptive plants that exploit innate and learned responses of animals by mimicking VOCs of food sources these animals seek without providing a reward (Galetti, 2002, Vereecken and McNiel, 2010). The VOC signals produced by plants to optimize pollination and seed dispersal and the way olfactory signals are perceived and interpreted by animals to find the most rewarding food sources evolved as a result of an evolutionary arms race driven by the self-interest of both partners. Temporal and spatial changes in the VOC emissions of flowers and fruits are orchestrated by a cascade of enzymatic reactions that are fine-tuned to prevent interactions with non-

beneficial interaction partners such as herbivores and microorganisms and to facilitate interactions with beneficial animal interaction partners (Muhlemann et al., 2014, Shari and Tahir, 2011). Food seeking flower visitors and frugivores should therefore be sensitive to temporal changes in the VOC composition because such changes are often correlated with a change in the nutrient content found in flowers and fruits. Pre-mature stages of flowers and fruits for example emit characteristic VOCs, so called green-leaf volatiles (GLVs), which might be used by flower visitors as info chemicals that signal that it is too early to find nutrients (e.g. nectar or sugary fruit pulp). Increased metabolism and production of nutrients or their decomposition is often accompanied by a change in the composition and/or emission rate of VOCs from plant tissue (Oikawa and Lerda, 2013, Xue et al., 2013). Post-mature stages on the other hand may emit decomposition VOCs (D-VOCs) and microbial VOCs (M-VOCs) that are indicative of decay, fermentation and conversion of nutrients into metabolic end-products. Volatile signals from fruits are usually intended for vertebrate dispersers and thus emitted at the ripe stage. Signals from late-stage fruits that are used by insects to find oviposition sites are more likely to be M-VOCs. One simple reason is that insects that oviposit on fruits are usually not specialized on particular plant species and thus M-VOCs rather than plant VOCs are likely to provide universal signals of over-ripe fruits.

Indeed plant reproductive parts harbour a high diversity of microorganisms such as bacteria, yeasts, and archaea (Alekkott et al., 2014). Thus the nutrient sources that plants produce as rewards for needed interaction partners are at risk to be exploited by microorganisms that occupy a commonly overlooked trophic level between flowers/fruits and mutualistic insects and vertebrates (Junker and Tholl, 2013). Microorganism might not only affect the interaction of plants with their mutualistic animal partners because they feed on rewards provided for pollinators and seed dispersers. In addition they might also interfere with the signalling because during the consumption and fermentation of floral nectar, flower tissue and fruit tissue characteristic microbial VOCs (M-VOCs) are released (Fridman et al., 2012). Such M-VOCs might affect the behaviour of mutualistic animal interaction partners as they may signal during the initial stages of decomposition and fermentation that nutrients are present, and during late stages that resources have been converted into metabolic end-products that are either toxic, of low energetic value to the animal, or depleted (Davis et al., 2013). Furthermore, microorganisms emit characteristic VOC patterns that are often indicative of the specific nutrient sources they are metabolizing such as sugar, fat or protein (Herrera et al., 2012, Janzen, 1977, Jürgens et al., 2013). Therefore, understanding what role microorganisms play in the interaction of mutualistic animals with

angiosperm flowers and fruit is of importance in a variety of contexts, including sensory ecology, evolution of pollination and fruit dispersal systems, and agriculture and pest control (Alekklett et al., 2014).

I start this review with a brief evaluation of the functional role and the evolution of plant volatiles with respect to different trophic interaction partners. I then examine the physiological processes responsible for flower and fruit decomposition and the role of plant regulated versus microbial processes in decomposition of plant tissue. Based on this I will compare the changes in the VOC composition of immature, non-active flowers and fruits, mature/ripe stages and old wilting/withering flowers and overripe decaying fruits may affect animal behaviour with respect to pollination and seed dispersal. Since information on the interference of microbes with plants and their mutualistic animals mainly concerns fleshy fruit and floral nectar, this review will focus on the volatile metabolic end-products of carbohydrate fermentation and tissue degradation by microorganisms and how they interfere with plant-animal communication.

1.2. Hypotheses for the evolution of VOCs in flowers and fruits

Insects, birds and mammals are the animal groups mostly involved in pollination and seed dispersal (Fleming and Kress, 2011). It is generally agreed that plant VOCs are important signals to attracting pollinators and seed dispersers, although there is far more evidence regarding the role of FL-VOCs in pollinator attraction than for that of FR-VOCs in attracting seed dispersers (see: (Knudsen et al., 2006, Rodriguez et al., 2013)). The general hypothesis for the co-evolution of angiosperms and their pollinators is that the association between beetles and angiosperms started already during the early Cretaceous period about 130 million years ago (Wang et al., 2013). Evidence for the association of angiosperms with a wider range insect groups and in particular with hymenopterans is the evolution of nectaries in late Cretaceous flowers about 99.6 to 65.5 million years ago (Wang et al., 2013). The interaction of pollinators with flowers seems to indicate adaptation or more generally a shared evolutionary history with angiosperms. Thus it is often assumed that features of angiosperm and their pollinators co-evolved as a result of the mutualistic nature of the relationship. However, more recent studies on the evolution of scent in different groups of angiosperms suggest that at least for some pollination systems ‘receiver bias’ may better explain the association between plants and their pollinators than co-evolution (Schiestl and Dötterl, 2012). The evolution of frugivory and seed

dispersal via vertebrates is according to Fleming and Kress (2011) at least 90 million years old. Herbivorous dinosaurs were perhaps seed dispersers before birds and mammals arrived on the scene but direct fossil evidence is very scarce (Fleming and Kress, 2011, Rodriguez et al., 2013, Tiffney, 2004). However, based on the fossil record it seems that the evolution of larger, fleshy fruits was probably mediated by more efficient avian and mammalian seed dispersers (Fleming and Kress, 2011).

Another trophic interaction level, the microbial level, is often overlooked when investigating plant-animal interactions (Davis et al., 2013). The bacterial communities that are found on flowers and fruits can be diverse and they are distinct from other microbial communities e.g. those found on leaves or in the soil ((Alekklett et al., 2014, Leff and Fierer, 2013) and references therein). On flowers these bacterial communities can differ between different species and also within species between different floral parts (see (Alekklett et al., 2014) and references therein). Already fresh fruits can harbour large bacterial populations of phylogenetically diverse phyla such as Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria (Badosa et al., 2008, Leff and Fierer, 2013). A high diversity of fungal taxa has also been reported for flowers and fruits. Tang et al. (2003) reported for example 102 taxa of fungi on 18 species of wild fruits in Hong Kong, and a metagenomics study using 18S rRNA sequences found fungi as the most prevalent community members on tomato flowers (Ottesen et al., 2013). Not all of these microorganisms contribute to flower and fruit decomposition. In fact for some of the microorganisms a mutualistic relationship with their plant host was suggested (Cipollini and Stiles, 1993). Some species of the microbial communities have been shown to protect the plant against pathogens in both flowers (Pusey et al., 2009) and fruits (Förnkrantz et al., 2012). For example, many endophytic *Saccharomyces* species that are present in fruit inhibit the growth of other pathogenic species by producing ethanol that is a metabolic end product of sugar fermentation (Levey, 2004). Since ethanol makes the medium bacteriostatic the relationship of plants with endophytic *Saccharomyces* might be mutualistic.

Almost 30 years ago it was hypothesized that floral volatiles evolved originally as a chemical defence against non-beneficial flower visiting insects and that their function changed during the course of evolution to attract pollinators (Pellmyr and Thien, 1986). Thus in addition to their attractive function plant VOCs may also act as filter mechanisms against antagonistic interaction partners that could be detrimental to the plant's reproduction including unwanted microorganisms (Herrera, 1982, Junker and Bluthgen, 2010). Antimicrobial and antifungal properties have been reported for many plant

VOCs such as terpenes (Farre-Armengol et al., 2013, Raguso, 2004, Steinebrunner et al., 2008). A similar evolutionary scenario has been suggested for the role of VOCs in fruits i.e. they were first produced as defence against herbivores and microorganisms and then later on their function changed to attract seed dispersing animals (Cipollini and Levey, 1997, Mack, 2000, Rodriguez et al., 2013, Tiffney, 2004). It has also been hypothesized that some of the VOCs produced by bacteria and fungi, such as organic acids and alcohols produced by yeasts, play a role as chemical defence mechanisms against other microorganisms (Janzen, 1977, Sherratt et al., 2006). Janzen's (1977) original idea that microorganisms produce chemicals, including VOCs, to render fruit and seeds objectionable to larger animals, thereby increasing the likelihood that they retain the resource, however, it seems questionable. It is possible that the chemicals produced by microbes have a deterrent effect on larger animals but this effect seems not to be the result of competition with animals and probably evolved in a different context. Using mathematical models Sherratt et al. (2006) found that if fruit spoiling carries a cost for the microbe, a likely evolutionary scenario, not even group selection can enable a spoiling strategy to persist. Because of the high dispersal rates of microbes it is very unlikely that producing fruit spoiling chemicals is an evolutionary advantageous strategy (Sherratt et al., 2006). Although speculative it is possible that the secondary metabolites that are found today in flowers and fruits have not only been selected for to prevent the growth of pathogenic microorganisms but also to allow the growth of microbes that have positive effect on plant survival and reproduction (Förnkrantz et al., 2012, Pusey et al., 2009). Studies showed that fruit flies are attracted to the VOCs that are produced by microbes, flies then visit and facilitate the dispersion of spores to other foods (Venu et al., 2014).

Microorganisms produce a wide range of VOCs and it is assumed that the olfactory systems of insects evolved features to exploit M-VOCs as behavioural cues long before the occurrence of angiosperms (Davis et al., 2013, Schulz and Dickschat, 2007). It is therefore likely that the interaction of insects with angiosperms and the olfactory communication between them is in fact to some extent the result of 'ecological fitting' (Janzen, 1977, Janzen, 1985) and more specifically 'receiver bias' (Schiestl and Dötterl, 2012). M-VOCs such as ethanol, CO₂, hydrocarbon acids, might provide reliable olfactory signals to carbohydrate seeking animals because they are directly derived from metabolised sugar (Goyret et al., 2008). It is known that flower visiting insects have very sensitive CO₂ receptors and they may use them to find flowers producing CO₂ due to respiration (Goyret et al., 2008). Typical fermentation volatiles such as ethanol, acetic acid, ethyl acetate, and acetaldehyde have been reported to attract several *Drosophila* species. In a study a mixture of five compounds (ethanol, acetic acid,

acetoin, 2-phenyl ethanol and 3-methyl-1-butanol) characteristic of VOCs emitted by *Saccharomyces* yeast was as attractive to naïve *D. melanogaster* as fermenting grape juice, suggesting yeast volatiles, not plant derived FR-VOCs were mainly responsible for their attraction (Becher et al., 2012). However, it is often not easy to identify which compounds are plant derived and which are due to the presence of microorganism. Plants produce ethanol, acetaldehyde, and acetic acid during primary metabolism (Oikawa and Lerda, 2013) and 2-phenyl ethanol can be produced by both yeasts and (Becher et al., 2012, Knudsen et al., 2006, Tieman et al., 2007, Goodrich et al., 2006). Some flowers attract their pollinators (mammals, fruit flies, and beetles) by producing typical microbial scent signatures very similar to yeast volatiles of fermenting fruits. Finally, carrion flowers produce microbial VOCs similar to that of decaying organic material to attract animals that are normally seeking carrion or dung as food- and/or brood (Jürgens et al., 2013, Vereecken and McNiel, 2010).

Floral microbial communities can have a direct effect on F-VOC emission by altering the scent emission, this was demonstrated by Peñuelas et al. (2014). The composition and proportion of terpenes in the floral scent of antibiotic-fumigated *Sambucus nigra* plants differed one week after the treatment from that of non-fumigated control plants (Peñuelas et al., 2014). Microbes may affect the interactions of flowers and fruits with food seeking animals also in two other ways: Firstly, microbes can convert nutrients so that they are of low energetic value, or even toxic to pollinators and seed dispersers (Herrera et al., 2008, Janzen, 1977). Such changes may also affect the taste or in the case of fruits the texture of the food source. Secondly, the emission of characteristic microbial VOCs may be used by animals as olfactory info chemicals for the presence or absence of nutrients (Davis et al., 2013, Laska and Seibt, 2002, Tomberlin et al., 2012). In consequence animals may learn to associate M-VOCs with a certain quantity and/or quality of reward. This can potentially lead to a conflicting relationship between plants and their mutualistic animals that provide transportation services to the plant, and microorganisms that may affect pollination service and fruit dispersal. However, very few studies have actually investigated whether microorganisms affect the relationships of plants with their mutualistic animal partners (Junker et al., 2014). Trophic interactions between plants, microorganisms living on plant tissue, and mutualistic animal partners that feed on floral tissue and floral rewards and fruits can be complex. While some studies have reported that microorganisms may affect plant-animal mutualisms negatively (Herrera et al., 2012), other studies found that the presence of microorganisms do not necessarily reduce plant-animal mutualisms (Vannette et al., 2013).

During the initial evolutionary phase VOCs were probably produced as a chemical defence against competitors or antagonists (in microorganisms probably against other microorganisms). Then later some of the interaction partners started utilizing these VOCs as info (information) chemicals. In angiosperms this has led to the evolution of relatively specific signalling pathways where different compounds in a VOC mixture can have specific or multiple functions. Accordingly some compounds are produced as animal attractants, others for their antimicrobial properties or as deterrents against herbivores (Huang et al., 2012, Junker and Bluthgen, 2010).

1.3. Senescence and post-senescence of flowers and fruits: physiological processes and trophic interactions with microorganisms

In comparison to leaves, flowers and fruits are ephemeral structures that last only for relatively short time periods. Based on phylogenetic analyses of protein sequences for colour pigments, Thomas et al. (2009) concluded that “phylogenetically and ontogenetically, the pigmented sepals, petals and dispersal structures of angiosperms are essentially heterochronic senescing leaves”. This idea is supported by the fact that the colour features of mature flowers and ripe fruits plants are the result of the same regulatory network used in leaf senescence (Thomas et al., 2009). Taking this idea further it would be interesting to compare their results with the regulatory mechanisms for the biosynthesis of VOCs in plants.

Noticeable is that the phenylpropanoid pathway to produce anthocyanin-based pigmentation in senescing leaves, in flower petals, and ripe fruits is also important for the production of second largest class of floral- and fruit VOCs namely phenylpropanoids/benzenoids (Knudsen et al., 2006).

Phenylpropanoids/benzenoids play an important role as signals to pollinators and seed dispersers (Borges et al., 2011, Knudsen et al., 2006). Among the VOCs produced by the phenylpropanoid pathway are for example the 2-phenylethanol, benzaldehyde, benzyl alcohol, phenyl acetaldehyde, methylbenzoate, only to name a few (Muhlemann et al., 2014).

Temporal changes in VOC emission may be the result of physiological senescence and ripening processes initiated by the plant and/or by microorganisms that decompose plant tissue. These processes are interlinked since changes in production of VOCs that have antimicrobial properties can affect the growth and composition of microbial communities that are living on flowers and fruits (Junker and Tholl, 2013). In addition the lifetime of floral and fruit structures may affect the microbial communities living on them. The microorganisms that play an active role in tissue decomposition saprophytic

species that are mainly involved in post-senescence decay can be distinguished from parasitic species that attack flowers and fruits before and during maturation. The physiology of flower senescence and fruit ripening and how it is genetically regulated has been investigated in some detail (Hadfield and Bennett, 1997, van Doorn and Woltering, 2008). Each phase of flower and fruit development and ripening involves specific gene activities and dramatic shifts in gene expression may occur (Handa et al., 2012, Shari and Tahir, 2011). Although progress has been made in the last 15 years regarding some of the key regulators of senescence, many questions remain. What complicates the picture is that no common regulatory mechanism has been identified thus far. It seems that different species regulate senescence in very different way (Rogers, 2006). Based on respiration patterns, climacteric and non-climacteric flowers and fruits can be distinguished. One of the best investigated triggers in the senescence of climacteric flowers and fruits is the volatile plant hormone ethylene, whereas in non-climacteric flowers/fruits these processes seem to be ethylene-independent (Barry and Giovannoni, 2007). Ethylene has been shown to regulate genes that are involved in the production of various enzymes responsible for both flower senescence and fruit ripening such as pectinases, amylases and hydrolases (Barry and Giovannoni, 2007, Lelièvre et al., 1997). These enzymes break down cell walls to soften the tissue, convert carbohydrates into simple sugars, and are involved in colour changes of the tissue by breaking down chlorophyll. During later stages of the senescence process and with the beginning of post-senescence decay microorganisms will then attack the host using extracellular lytic enzymes (e.g. pectinases and hemicellulases) that degrade the plant tissue to access plant nutrients (Barth et al., 2010, Miedes and Lorences, 2004). It is therefore not easy to disentangle the plant regulated decomposition processes from added decomposition by microorganisms. Since the diversity and composition of microorganisms on plant tissue also depends on the type of tissue (Alekklett et al., 2014), therefore the senescence and decomposition of flowers and fruit are described separately.

1.3.1. Flower senescence

Flower senescence is a process leading to cell and tissue death of entire flowers or floral tissue not needed for fruit development. Flower senescence is a highly integrated process in which proteins are actively synthesised for the execution of cell death. Many of the senescence-enhanced genes in petals encode for catabolic enzymes that are involved in the breakdown of cell organelles and macromolecules (Shari and Tahir, 2011, van Doorn and Woltering, 2008). The senescence process

includes deterioration of cellular membranes and autophagy followed by remobilization of essential nutrients from senescing floral parts to developing floral parts (i.e. fruit parts) (Chapin and Jones, 2007, Chapin and Jones, 2009). In many species pollination has been identified as an important trigger of floral senescence (Rogers, 2006). Pollination induced flower senescence allows the removal of the structures no longer necessary (e.g. pollinator-attracting petals) so that nutrients can be redistributed to the growing ovary or other parts of the developing fruit. Other advantages of early senescence of the petals may be to decrease the attraction to potential herbivores (Webb and Littleton, 1987) and to increase the efficiency of the pollination process by reducing pollen loss to already pollinated flowers. In addition flowers can be attacked by pathogenic microorganisms that use the stigma to enter the plant (Shykoff et al., 1996). Thus closing these entry points after pollination may reduce the risk of a pathogen attack.

Three main external symptoms of flower senescence (or senescence of flower parts) can be distinguished: (1) turgor loss of petals leading to wilting, (2) colour change of petals due to the slow dehydration, and (3) abscission of dried or dead tissue or organs, often along predefined lines of dried dead tissue. With respect to un-pollinated or unsuccessfully fertilized flowers many species will start a natural wilting process and finalize it sometimes with abscission of the entire unsuccessful reproductive organ. Depending on the species natural senescence may be delayed in un-pollinated flowers compared to pollinated ones. Pollination-induced senescence has been shown in several species. Here flower senescence can already be induced by pollen deposition or pollen removal (reviewed by van Doorn, 1997). Woltering and Harren (1989) demonstrated in *Cymbidium* orchids that the removal of pollinia (pollen packages) triggers the senescence of the flower due to desiccation of the tissue of the rostellum that in turn stimulates the production of ethylene. Pollinated flowers with successfully fertilized ovules will go through a flower senescence and fruit maturation process including wilting, permanent flower closure, withering, and abscission of particular floral organs and tissues and development and maturation of others (i.e. ovule to embryo, ovary to seed, floral parts to fruit). These processes of flower senescence and fruit ripening are not necessarily in sequence; fruit ripening can start while wilting or withering floral parts are still attached to the developing fruit. Nevertheless the sequence and duration of the different stages are probably under selection so that the visual and olfactory features of flowers and fruits do not interfere with each other. Furthermore, it is likely that the different symptoms and sequences of events during flower senescence and fruit development or the lack thereof reflect

phylogenetic constraints, particularities of the reproductive system or adaptations to environmental factors (see van Doorn, 1997).

In their review Shahri and Tahir (2011) distinguished between five general patterns of petal senescence depending on how flowers respond to ethylene (ethylene-sensitive vs. ethylene insensitive plants), whether the wilting process is slow or fast, and whether abscission of petals can be observed. The response to endogenous ethylene in flowers, initiating senescence, varies widely. In many plant species petal wilting occurs without any apparent involvement of ethylene (Reid and Wu, 1992, Woltering and Van Doorn, 1988). Furthermore, the response to ethylene seems also to depend on whether the initial symptom of senescence is abscission or wilting (Shari and Tahir, 2011). Generally, in species exhibiting abscission a high sensitivity to exogenous ethylene was reported whereas species which typically wilt without abscission were not sensitive to ethylene (Reid and Wu, 1992 and references therein).

1.3.2. Fruit ripening and decomposition

During fruit ripening a dramatic shift in gene expression is observed. This results in many changes, often related to fruit dispersal, such as accumulation of sugar, reduction in organic acids, alterations in colour, and production of characteristic volatiles. Depending on whether the fruit is climacteric or not the fruit ripening process and VOC emission may differ (Barry and Giovannoni, 2007). Climacteric fleshy fruit are characterised by a quick onset and completion of fruit ripening (including tissue softening, and increase of sugar content) as a result of a rise in ethylene production whereas non-climacteric show gradual ripening and no sharp rise in ethylene (Barry and Giovannoni, 2007, Brady, 1987). Although ethylene is the best investigated phytohormone other compounds such as abscisic acid, auxin, brassinosteroids, gibberilins, and jasmonates have also been reported to be involved in fruit development and ripening (Srivastava and Handa, 2005). Furthermore, emission of FR-VOCs peaks sharply in climacteric fruit (e.g. tomato) but not in non-climacteric fruit (e.g. strawberry, grape, citrus). As pointed out by Borges et al. (2011) these differences between climacteric and non-climacteric fruit may also determine the type of VOCs produced and in consequence the type of dispersal agent that is attracted to it.

The sugars that are produced in many fruits with fleshy tissue serve as a reward to attract frugivores which disperse the seeds but they are also the main substrate for fermentation by microorganisms (Dudley, 2000). While most of the bacteria form a protective layer against pathogens that attack the fruit, some of them produce extracellular enzymes to get access to the nutrients contained in the fruit and causing the fruit to rot (Leff and Fierer, 2013). Indeed carbohydrates in the form of nectar and fruit tissue provide ideal conditions for the survival and growth of bacteria and yeasts. The activity of microorganisms results in the break-down of cell membranes and essentially the consumption of sugars and fruit tissue by microorganisms via aerobic and/or anaerobic pathways (Rogiers et al., 1998). Some authors have suggested that fruit are more vulnerable to the attack by microorganisms once they have ripened since ripening increases the nutritional value but it also reduces the chemical defences, e.g. by reducing the amount of organic acids, that prevent bacteria and fungi from feeding the sugars (Valburg, 1992, Dudley, 2000).

1.4. Temporal changes in VOC emissions of flowers and fruits and their role in mediating interactions with animals

Floral volatiles (FL-VOCs) and fruit volatiles (FR-VOCs) are characterized by a wide range of chemicals (El Hadi et al., 2013, Knudsen et al., 2006). Floral and fruit VOCs can be divided into the following major chemical classes that are commonly reported: (a) terpenoids (including monoterpenoids, sesquiterpenes, and irregular terpenoids), (b) phenylpropenoids and benzenoids, (c) fatty acid derivatives (e.g. hydrocarbon aldehydes, hydrocarbon esters, hydrocarbon alcohols, and hydrocarbon acids), (d) nitrogen containing compounds, and (e) sulphur containing compounds. Microorganisms are capable to produce all compound classes that have been reported from flowers and fruits (for a review see Schulz and Dickschat, 2007). However, especially widespread among bacteria are heteroaromatic compounds (e.g. pyrazines, indole), sulphur containing compounds (e.g. oligosulphides), geosmin (sesquiterpene), and 2-methylisoborneol (sesquiterpene) (Schulz and Dickschat, 2007).

It is difficult to clearly distinguish between FL-VOCs and FR-VOCs. FR-VOCs are often dominated by high relative amounts of hydrocarbon esters and benzenoid esters (e.g. banana, kiwi fruit, apple), and terpenoids (e.g. citrus), although FL-VOCs can contain the same compounds (El Hadi et al., 2013, Knudsen et al., 2006, Rodriguez et al., 2013). Temporal changes in scent emission and

composition may occur within or between different developmental stages of the reproductive organ (**Figure 1**). Changes in volatile composition are due to the activity of different enzymatic systems that are regulating these processes. For example, fatty acids are major precursors of hydrocarbon esters mediated by β -oxidation as the main metabolic pathway in the early stage of fruit development (Sanz et al., 1996). During the later stages β -oxidation is accompanied by the lipoxygenase system (LOX) (often together with the rise in ethylene production) leading to the formation of esters and C6 alcohols (Suárez and Duque, 1992). The LOX activity then declines as the fruit softens.

There is only limited knowledge about the role of temporal changes of plant VOCs for attracting pollinators and seed dispersers (e.g. Schiestl and Ayasse, 2001). From an animals' point of view VOCs may not only signal when it is best to use a resource (attractant) but also when the resource is not suitable (repellent). Such olfactory signals that indicate to an animal that a resource is depleted or unpalatable may either originate from the plant itself, e.g. as floral post-pollination VOC signal (Theis and Raguso, 2005) that directs effective pollinators to flowers still in need of pollination, or from flower visitors that leave scent marks on flowers indicating that the resource has recently been depleted (Wilms and Eltz, 2008). A third potential source for olfactory signals are microorganisms that may be transferred to flowers and fruits by animal visitors (e.g. nectar yeasts by flower-visiting insects) and may or may not compete with animals for carbohydrate rich resources in flowers and fruits (Leff and Fierer, 2013). In the following I characterize the general changes in plant VOC emission patterns for the different phases of flowers and fruits and discuss their role for interacting with animals. I also discuss the potential role of microbial communities for interfering with pollinators and seed dispersers.

Pre-flowering (bud) stage – Green-leaf volatiles (GLVs) are the dominant constituents of the pre-flowering stages of flowers and unripe fruit. Short-chained (C6) acyclic aldehydes, alcohols and their esters (e.g. (Z)-3-hexenal; (Z)-3-hexen-1-ol; (Z)-3-hexenyl acetate) are responsible for the to the human nose fresh, green-leaf like smell in bud stages of flowers and later again in many unripe fruits. In leaves GLVs are produced from linoleic acid and linolenic acids by the enzyme lipoxygenase (LOX). The C6-alcohols are formed from their corresponding aldehydes by alcohol dehydrogenase. Pre-flowering stages are normally not attractive for pollinating insects because it is assumed that they lack the typical VOC signature associated with the presence of resources such as nectar or pollen (Knudsen et al., 2006). In addition, compared to fully open flowers bud stages often have no or only a very small visual

display so that it is difficult for insects to distinguish them from foliage. Another possible explanation for the low attractiveness of bud stages is that some of the GLVs might act (learned or innate) as repellents that indicate to pollinators not to land. Interestingly GLVs have been shown to be the key attractants in a rare chemical mimicry system. The flowers of two *Epipactis* orchids *E. helleborine* and *E. purpurata* attract social wasps (*Vespula germanica* and *V. vulgaris*) as pollinators (Brodmann et al., 2008). Despite a large nectar reward, these flowers are overlooked by other flower visiting insects, whereas wasps that probably use GLVs to locate prey, e.g. caterpillars feeding on leaves, were attracted.

Pre-pollination flowering stage – The flowering stage is characterized by the advertisement of floral rewards via olfactory and visual signals. FL-VOCs are formed from precursor compounds that are produced before flower-opening (Watanabe et al., 1993). During flower opening newly induced or activated enzymes then mediate the terminal reactions of the precursor compounds (Watanabe et al., 1993). The emission of FL-VOCs can be up- and down- regulated so that emission and composition of FL-VOCs are synchronised with the activity time of the main pollinators (Dötterl et al., 2012, Dötterl and Jürgens, 2005). There are two main advantages for plants to restrict their VOC emissions to a limited time period not only during their life but also during the day or night. Firstly, the production and release of VOCs during periods when they have highest biological activity, e.g. as herbivore defence or pollinator attractants, conserves resources. Secondly, emitting VOCs for short time periods may prevent visitation by less efficient pollinators and florivores.

Flowers harbour unique microbial communities that are distinct in their composition and diversity from other anatomical parts such as leaves (Alekkett et al., 2014, Junker et al., 2011). Some bacteria and yeasts of the often complex microbial communities found on flowers have been shown to be antagonists of pathogenic microorganisms (Pusey et al., 2009). More recently a focus of interest has been on the microbial communities that can be found in nectar and how they influence pollinator behaviour and in consequence plant reproductive success (Herrera et al., 2008, Junker et al., 2014, Vannette et al., 2013). Yeast and bacteria can change the physicochemical features (e.g. pH, total sugar concentration, glucose and fructose concentration) of floral nectar and this in turn can alter the behaviour of pollinators (e.g. Herrera et al., 2008; Vannette et al., 2013; Junker et al., 2014). A recent study by Junker et al. (2014) suggests that bumblebees are able to perceive bacteria in densities similar

to that found in flowers and nectar. They also showed strain-specific density-dependent avoidance behaviour. As stated earlier such interference of microbes with pollinators and the potential negative effect on seed set may explain why plants have evolved defence mechanisms against bacteria, including chemical defence via VOCs, to inhibit microbial growth (Junker et al., 2014, Junker and Tholl, 2013). In a study by Vannette et al. (2013) the presence of nectar bacteria (*Gluconobacter* sp.), but not nectar yeast (*Metschnikowia reukaufii*) negatively affected seed set in *Mimulus aurantiacus*. Finally, Herrera et al. (2013) found that *Bombus terrestris* bumblebees preferred artificial sugar solutions with yeasts (*Metschnikowia reukaufii*) and yeast containing nectar of *Helleborus foetidus* flowers over artificial sugar solutions and flowers without yeasts. Furthermore they found that the modified pollinator foraging patterns had a negative effect on fruit set and seed set of *H. foetidus* (Herrera et al., 2013). All these studies suggest that the effects of microorganisms on the reproductive success of plants are not necessarily negative and that different microbes may have different effects. The effect of one compound, ethanol, on flower visitor behaviour has been studied in more detail. The concentration of ethanol in fermented floral nectar can reach values up to 3.8% as has been reported in the *Eugeissona tristis* which is commonly known as bertram palm (Wiens et al., 2008), however, normal concentrations are less than 1% (Abramson et al., 2004a, Abramson et al., 2005, Abramson et al., 2004b).

Post-pollination flowering stage – Most floral scent research has been focussed on the pre-pollination flowering stage and the olfactory cues that are involved in attracting pollinators. However, post-pollination changes in VOC emission or late stage changes could have more important implications for pollinator behaviour than previously thought. Several studies have shown that flowers may change their volatile composition after pollination thereby reducing their attractiveness to flower visitors (Schiestl and Ayasse, 2001, Theis and Raguso, 2005). This is either a mechanisms to increase the effectiveness of the pollination process or to reduce the predation risk of flowers by herbivores. The physiological mechanisms involved in post-pollination changes of FL-VOC emission have been investigated in a few cases. Ethylene has been reported by Negre et al. (2003) to shut down emission of methylbenzoate in petunias after pollination. However, the same authors found that snapdragons use a different mechanism to suppress or decrease methylbenzoate emission. In snapdragons flower scent emission is regulated by a change in the ratio of two chemicals SAM (S-adenosyl-L-methionine) and SAH (S-

adenosyl-L-homocysteine). In both species a down-regulation in floral scent begins only after fertilization (Negre et al., 2003) thereby guaranteeing that the attraction of flower visitors via scent is maintained until pollen has successfully fertilized ovules.

Decomposing flowers, flower withering/wilting/abscission and onset of fruit development –

Although it has been reported from several studies that in senescing flowers the scent emission weakens and also the VOC composition changes (Schade et al., 2001) there is still a gap in our knowledge whether such changes play an ecological role. It seems possible that the behaviour of pollinators is influenced by volatile emissions from floral parts that are starting to wilt or wither either due to senescence processes induced by the plant or due to microbial decay of plant tissue. A study by Schade et al., (2001) showed that different floral stages of carnation flowers (*Dianthus caryophyllus* L.) had distinct proportions of the ten VOCs produced by the plant. The compounds benzaldehyde, benzyl alcohol, and (*E*)-caryophyllene were higher in late stage senescing flowers compared to earlier stages (Schade et al., 2001).

Unripe fruits – The primary function of terpenoids (monoterpenes, sesquiterpenes, irregular terpenes, and homoterpenes) and GLVs in immature fruit is to defend against potential consumers such as microorganisms and pre-dispersal seed and pulp predators, that could negatively affect plant reproduction (Cipollini and Levey, 1997). The VOC composition changes during maturation to attract fruit and seed dispersers. In fruits such as nectarines, apples, guava, kiwi, and strawberries the concentration of GLVs compounds decreases with ripening (Rodriguez et al., 2013). A study by Xue et al. (2013) showed that the energetic investment of plants in producing FR-VOCs can lead to a significant decrease in nutrients. The authors reported two shifts in the carbon-nitrogen metabolism during seed development of *Nigella sativa*). The first shift, an increased level of sugars and sugar alcohols, was observed during the maturation of seeds (Xue et al., 2013). These metabolites are precursors of the triacylglycerol metabolism suggesting they are used for the accumulation of fatty acids as storage resources (Xue et al., 2013). The second shift was characterized by the biosynthesis of VOCs and a 30% average decrease in total fatty acids (Xue et al., 2013).

Ripe fruits – FR-VOCs have multiple functions: (1) they can attract frugivores that act as seed dispersers (2) they can be a chemical defence against microorganisms, and (3) they can be a chemical defence against herbivores that destroy seeds. A recent review by Rodríguez et al. (2013) on the role of VOCs in mature seeds and fleshy fruits as signals for seed dispersers revealed that our understanding of the function of VOCs wild fruit is still very limited and that most of our knowledge is based on horticultural and crop species. One of the few examples is a study by Borges et al. (2011) that reported differences in the FR-VOC composition of *Ficus benghalensis*, a species with a long ripening period that is dispersed by birds during the day and bats during the night. The authors hypothesized that the emission of fatty acid derived esters and benzenoids during night and sesquiterpenes during the day might be related to the different seed dispersers of *F. benghalensis*. This suggest that similar to what has been observed in some flowers fruit may also show temporal changes in the FR-VOC emission and composition to attract different seed dispersers during day and night.

As stated earlier the relationship between plants and certain microorganism feeding on fruits has been considered as predatory because microorganisms that consume fruits make them unattractive to vertebrate frugivores (Dominy, 2004, Levey, 2004). The traditional view has therefore been that animal dispersers of fleshy fruit compete with microorganisms for food resources and that FR-VOCs play a role as antimicrobial defence mechanism (Herrera, 1982, Janzen, 1977). Accordingly plants should produce secondary metabolites, including FR-VOCs, that prevent or reduce the growth of microorganisms but at the same time have a minimal effect on seed dispersers (Buchholz and Levey, 1990, Levey, 2004, Tewksbury et al., 2008). However, a study by Rodríguez et al. (2013) on the role of FR-VOCs emitted by orange (*Citrus sinensis*) fruit peel contrasts with this view. They found that transgenic orange plants with reduced accumulation of limonene in the peel showed marked resistance against two pathogens, the fungus *Penicillium digitatum* and the bacterium *Xanthomonas citri*. This suggests that the tritrophic relationship between microbes, plants and their animal seed disperser are more complex than previously thought. Although inhibitory effects of monoterpenes, including limonene and derivatives, have been reported (Ben-Yehoshua et al., 2008) citrus FR-VOCs have also been shown to play an important role in host recognition by *P. digitatum* (Droby et al., 2008). According to Rodríguez et al. (2013) the interaction of *C. sinensis* with microorganisms that make the fruit accessible for dispersal by mammals is a necessary first step to break -down the toxic terpene-rich peel barrier. As a result of infestation by microbes the terpene content of the fruit would drop and be less deterring to terrestrial mammals that could then act as seed disperses (Rodríguez et al., 2013).

Overripe (decomposing) fruits – The odours of overripe/fermenting fruits are usually associated with decay. Common VOCs found in fermenting or overripe fruits include hydrocarbon alcohols- and esters such as ethanol and ethyl acetate (Levey, 2004). Methanol is not only a product of fermentation but also produced by the plant enzyme pectin methylesterase that is involved in fruit softening (Lefever et al., 2004). Ethanol is usually an end product of anaerobic fermentation (Levey, 2004) whereas aerobic pathways via oxidation of ethanol result in acetic acid. Sugars can oxidise completely to CO₂ and H₂O or only partially to organic acids and alcohol. The ethanol content may differ between lipid rich and sugar rich fruits and ethanol is normally higher in sugar rich fruits (Dominy, 2004).

Very little is known whether M-VOCs emitted by overripe fermenting fruit are identifiable by vertebrates (see Rodríguez et al., 2013 for a review). Animals approaching overripe fruit are exposed to a blend of fruit and microbial-derived odours. It is therefore not so easy to identify whether frugivores respond to M-VOCs, FR-VOCs or both. In general, primates, rodents and bats are sensitive to fruits infested with microorganisms (Dominy, 2004, Levey, 2004) and depending on their trophic preferences animals are attracted to different ripening stages of fruits. Rodríguez et al. (2013) pointed out that although most birds and small mammals prefer ripe, uninfected fruits some specialized rodents prefer fruits that show signs of decay (Borowicz, 1988, Cipollini and Stiles, 1993). However, overripe fruits rich in ethanol would probably also be consumed by vertebrates in situations when there is no alternative food resource (Dominy, 2004, Sánchez et al., 2008). There has been a particular interest in the evolutionary ecology of ethanol consumption by fruit dispersers and also pollinators because of its implications for the evolution of alcoholism in humans (Levey, 2004). In primates the production of ethanol in fruits along with the soft fruit texture is used as information on fruit quality (Dominy, 2004). A study carried out suggests that fruit-eating squirrel monkeys and pigtail macaques are sensitive to the scent of aliphatic alcohols (Laska and Seibt, 2002). Sánchez et al.(2006) detected ethanol, acetaldehyde, and acetic acid as the only fermentation volatiles emitted by two fruit-bat dispersed species *Phoenix dactylifera* (dates) and *Ficus rubiginosa* (rusty figs). When they tested the Egyptian fruit bat (*Rosettus aegyptiacus*) for their response to ethanol they found that ethanol concentrations (>1%) higher than those found in ripe fruit were avoided by the bats (Sánchez et al., 2004). This suggests that ethanol at higher concentrations are used as an odour cue to bats to avoid fruits that are overripe and unpalatable (Sánchez et al., 2006). Sánchez et al., (2006) also tested the effect of methanol on fruit bats by mixing it in water or mango juice. Contrary to their expectations they found that the

smell of methanol had no effect at any concentration. These findings are surprising given the toxic effects of methanol on vertebrates (Sánchez et al., 2006 and references therein).

1.5. Conclusions and future perspectives

Although many studies in the last five years have started to look into the trophic interactions of microorganisms with floral- and fruit- structures more research is needed to understand how they affect volatile emissions and interactions with pollinators and fruit dispersers. Although the presence of microbes that are exploiting the resources provided by flowers and fruit has been hypothesized to have a negative effect on pollination and fruit dispersal, this may not apply to all cases or to all microbes (Rodriguez et al., 2013, Vannette et al., 2013). The available information on this topic strongly suggests that the traditional bi-trophic, plant–pollinator and plant–seed disperser concepts must be updated to accommodate for the role of microorganisms in plant-animal interactions. I agree with Davis et al. (2013) —.that insect olfactory responses to emissions from microorganisms inhabiting their sensory environment are much more common than currently recognized, and that these signals represent evolutionarily reliable info chemicals.” A more integrated approach to volatile signals including those produced from other trophic interaction partners like e.g. bacteria and fungi is therefore needed. Only moderate knowledge whether the production of M-VOCs provides a selective advantage for microorganism and what the potential ecological functions are (Janzen, 1977, Sherratt et al., 2006). In addition to their antimicrobial function discussed earlier some of the volatile compounds produced by bacteria might for example be involved in intra- and interspecific communication (quorum sensing). Another possibility is that M-VOCs are produced to attract dispersal agents such as fruit flies and other insects (Sang, 1956; Ganter, 2006). Targeted dispersal has been suggested for yeasts living on sugar-rich fruit substrates and also for nectar yeasts (Reuter et al., 2007).

I think that the traditional approach of distinguishing between typical flower, fruit and microbial volatiles is difficult to maintain. Because both plants and microorganism can produce a high diversity of volatile chemical compounds there is considerable overlap in their volatile chemistry. Interesting examples are flowers that attract pollinators that normally utilize fruits and seeds as food and brood sites such as beetles (Jürgens et al., 2000, Steenhuisen et al., 2013), fruit flies (Stökl et al., 2010), and mammals (Johnson et al., 2011). Another example are carrion flowers that use microbial info chemicals to attract sapromyiophilous flies or beetles that use carrion as food and brood sites (Jürgens et al.,

2013). Furthermore, there are also fungi that imitate the volatiles emitted from carrion (Johnson and Jürgens, 2010), flowers that imitate the VOCs of fungi (Kaiser, 2006), and fungi that emit the typical VOCs of flowers (Raguso and Roy, 1998). All this indicates that different organisms are very flexible regarding their VOC chemistry but it also indicates that some volatile combinations represent universal info chemicals used by animals.

To find food sources animals should combine VOC information from different sources including those emitted by microorganisms because they are often found at potential food sources. M-VOCs from bacteria and fungi might be particularly useful to animals because they are directly related to the presence macronutrients such as carbohydrates (fermentation volatiles), protein (oligosulfides and nitrogen containing compounds). M-VOCs might even provide more reliable signals on the nutritional status of a flower or fruit than the signals emitted by the plant. Unfortunately, there is still too little evidence on M-VOCs and how they influence plant-animal interactions to draw any general conclusions on the topic.

There are three main gaps in our knowledge regarding the role of VOCs for the interaction with mutualistic animals. Firstly, the ecological role of FR-VOCs for attracting fruit dispersers has only been investigated in very few cases (Rodriguez et al., 2013). Most of our knowledge on FR-VOCs is based on horticultural varieties and more research is needed on wild fruit (Borges et al., 2008, Borges et al., 2011). Secondly, VOCs emitted by microorganisms that feed on nectar and flower/fruit tissue could play a role as additional signals to animals that act as pollinators and food dispersers. It is therefore necessary to disentangle plant derived floral and fruit VOCs from microbial derived VOCs. Finally, to our knowledge there are no studies that compared the volatile emissions and biosynthetic pathways used of a plant during the whole life cycle including flowers, fruits and vegetative parts. The vast majority of studies that are interested in the ecological function of floral and fruit VOCs have focused on the active stages and the compounds that attract animals. It is possible that VOCs from pre-mature and post-mature stages play an equally important role for animals to optimize their foraging behaviour and for plants to optimize the ~~use~~ of their animal vectors.

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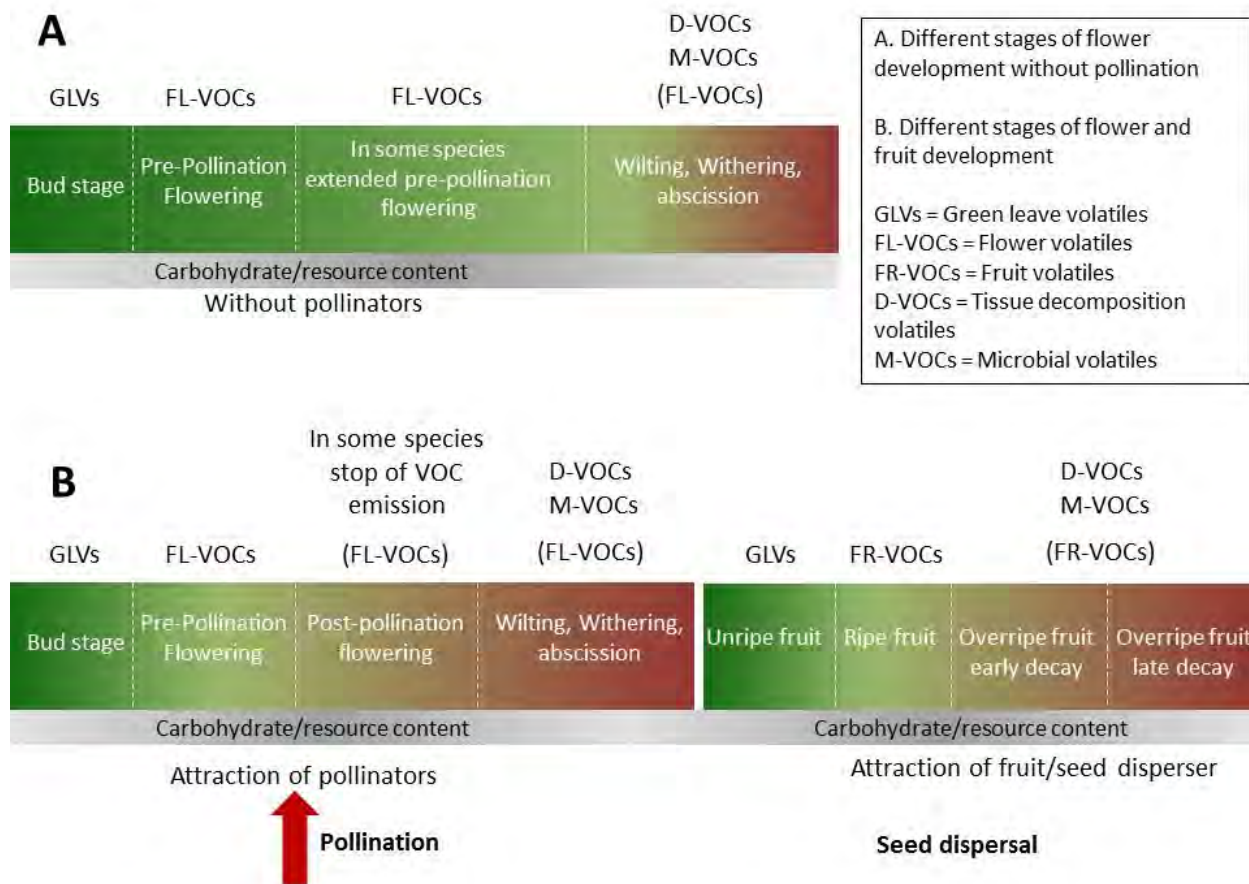


Figure 1. Temporal sequence of flower and fruit development and the different types of volatile organic compounds (VOCs) typically emitted at each stage. **A.** Development of an un-pollinated flower. **B.** development of a pollinated flower. (1) Pre-flowering (bud-stage), (2) pre-pollination flowering, (3) post-pollination flowering, (4) flower withering/wilting/abscission and onset of fruit development, (5) unripe fruiting, (6) ripe fruiting, (7) overripe fruiting (early decay), 8 overripe fruiting (late decay). Changes in the carbohydrate content and the potential for interacting with animals. Green indicates early stages characterized by the emission of GLVs, whereas red indicates late stages characterized by senescence and the emission of D-VOCs and M-VOCs.

Chapter 2

A comparison of Solid Phase Micro-Extraction (SPME) and Micro Solid Phase Extraction (Micro SPE) as techniques for collecting typical fermentation volatiles

Abstract

Flowers and fruits emit an array of compounds often characterized by many highly volatile hydrocarbon alcohols and hydrocarbon acids with low molecular weight. These compounds are, however, often difficult to detect with conventional headspace extraction techniques that use solvents. This is a particular problem for researchers interested in fermentation volatiles because many of the emitted compounds (e.g. ethyl acetate, acetic acid, acetoin) overlap in their retention time with solvents that are commonly used for extracting these compounds such as hexane, acetone, or dichloromethane. There has therefore been an interest in alternative methods that avoid solvent extraction such as direct trapping of target molecules on an absorbent followed by either thermal desorption in conjunction with an intermediate cold trap or the more cost effective method of direct thermodesorption in the injector of the gas-chromatograph. Here, two commonly used direct thermodesorption methods for the collection and release of volatiles, namely: solid phase micro-extraction (SPME) and micro solid phase extraction (Micro SPE) were compared. The sensitivity of SPME and Micro SPE was investigated by using standard mixtures of floral- and fermentation volatiles typically found from decomposing plant material. The SPME and Micro SPE samples were subsequently analysed via gas chromatography coupled with mass spectrometry (GC-MS). The results indicated that both Micro SPE and SPME were effective in collecting floral and fermentation volatiles. There was a significant difference in the absolute emission of compounds when using SPME, and Micro SPE with a DB5 and Carbowax column. There were significant levels of bleed-through of compounds of lower molecular weight when using Micro SPE.

Keywords: Volatiles, flowers, fruits, fermentation, SPME, Micro SPE and GC-MS.

2.1. Introduction

Understanding the function of VOC emissions for the interaction of plants with other organisms such as pollinators and seed dispersers has become an important aspect of ecological and evolutionary research. Flowers and fruits produce and emit various volatile organic compounds (VOCs) which facilitate interspecies interactions (Baldwin, 2010). There is a high diversity of VOCs emitted during the life cycle of both flowers and fruits (Knudsen et al., 2006). A challenge, however, is the collection and identification of fermentation volatiles that are emitted during the senescing stages of flowers and fruits. The reason is that VOCs of these stages include several relatively short chain hydrocarbon compounds (esters, acids, and alcohols) with low molecular weight. Thus, solvent extraction methods that use e.g. hexane, acetone, or dichloromethane (DCM) are not suitable for the detection and identification of these compounds because the solvent peak overlaps with the early retention time of many compounds that are key indicators for fermentation such as ethyl acetate or acetoin. Solvent methods also have a lower sensitivity because only a fraction of the eluted solvent can be injected into the gas-chromatograph (GC) and any attempts to reduce the volume of solvent, such as blowing them down with nitrogen, usually result in the biased loss of low molecular weight volatiles. To solve these problems several solvent-free methods are now available that use an adsorbent, e.g. in the form of a fiber or a powdered polymer contained within a small vial, that is then directly introduced into the injector of the GC. Methods where volatile compounds binding to the adsorbent are released directly onto the column by heating up the injector, as opposed to passing through an intermediate cold-trap, are known as direct thermodesorption methods. Direct thermodesorption is a relatively cost effective method because it does not require the complex cryo-system associated with a cold trap. However, not much is known about the relative performance of different methods of direct thermal desorption.

Here, two more commonly used thermodesorption methods were selected for comparison because both methods have been used more recently to investigate fermentation VOCs emitted during the late stage of flowers and fruits (e.g. Flamini et al., 2003; Li et al., 2006; Dötterl and Jürgens, 2005; Jürgens et al., 2006, 2010, 2012). These methods are: solid phase micro-extraction (SPME), (Arthur and Pawliszyn, 1990) and micro solid phase extraction (Micro SPE) (Amirav and Dagan, 1997). The technique used for the collection of samples may play an important role since fermentation volatiles are often emitted only in trace amounts and applying the most sensitive technique is therefore desirable. Both SPME and Micro SPE are used in conjunction with gas chromatography mass spectrometry (GC-MS) for subsequent analyses of volatiles.

SPME is a passive sampling method which does not use air samplers/pumps for collecting volatile compounds. Numerous studies have utilized SPME as an extraction method. SPME incorporates the steps of sampling, extraction, and sample introduction into a single solvent-free step (e.g. Vas and Vékey, 2004). Headspace SPME facilitates the attainment of an equilibrium between the sample matrix, the headspace above the sample and a static phase which is coated on a fused silica fiber. Currently, SPME is commonly used in combination with GC-MS (Li et al., 2006, Vas and Vékey, 2004). SPME is a fast, effective, sensitive solvent free sampling method. The applicability of SPME is measured using the octanol-water distribution coefficient (K_{ow}), which determines how well a solute can be extracted using SPME (Hyötyläinen, 2009). The absorption ratio is also based on the thickness of the coating. In order for SPME to be quantitative, solutes need to have a high $\log K_{ow}$ value, generally above ca. 5 (Hyötyläinen, 2009). According to Li et al. (2006) SPME is a commonly used extraction method because it avoids chemical alteration and formation of artifacts that occur in conventional solvent methods. Findings by Flamini et al. (2003) indicate that SPME facilitates the detection of a wide variety of compounds and that it requires relatively short sampling times and thereby removing artifacts that can occur, e.g. due to plant damage (Flamini et al., 2003).

There is a growing popularity of using SPME. For instance Yuan et al. (2014) used SPME to determine the emission of VOCs from water lily flowers (*Nymphaea*). Flowers of both the groups were collected between 5:00 am and 7:00 am and 2.5g of petals and 0.5g of stamens and pistils were sampled using headspace SPME with a PDMS (100 μ m) fiber (Yuan et al., 2014). In total 117 compounds were detected and it was found that the highest concentration of compounds were found in the stamens of the water lilies (Yuan et al., 2014). In another study the emission of VOCs from individual parts of *Citrus deliciosa* were investigated (Flamini et al., 2003). Samples were collected from the whole flowers, petals, stamens, gynoecium and pollen using a 100mm PDMS fiber. SPME resulted in the detection of a range of VOCs between all flower parts. Furthermore, pollen scent largely contributed to the whole flower scent (Flamini et al., 2003). These two examples illustrate that SPME has been used successfully to detect temporal and spatial scent patterns in flowers. In a study carried out by (Li et al., 2006) to determine the VOCs emitted from two varieties of *Syringa oblata* flowers, three different SPME fibers were used to determine which one is the best to collect the floral VOCs. This is a critical point because several fibers with different chemical properties for trapping VOCs are available for SPME. The fibers included in their study were: (i) 65 μ m polydimethylsiloxane/divinylbenzene (PDMS/DVB), (ii) 30 μ m polydimethylsiloxane (PDMS) and 65 μ m Carbowax/

divinylbenzene (CW/ DVB). It was found that PDMS/DVB fiber was more efficient regarding its extraction efficiency than PDMS and CW/DVB. The peak areas of the major compounds were also higher with PDMS/DVB (Li et al., 2006).

The second thermodesorption method that has been used for the identification of compounds with low molecular weight and early retention times is Micro SPE (Amirav and Dagan, 1997). Micro SPE uses dynamic headspace and is regarded as an active sampling method. It entails the use of thermodesorption tubes filled with absorbents such as Carbotrap or Tenax, which are responsible for the trapping of volatiles (Van der Niet et al., 2010, Amirav and Dagan, 1997). A battery operated micro-air sampler/pump is used to pump the scent containing air through and the glass vials filled with an adsorbent (Dötterl and Jürgens, 2005, Dötterl et al., 2005, Jürgens et al., 2012, Jürgens et al., 2010, Jürgens et al., 2009, Van der Niet et al., 2010). The use of Micro SPE has grown in popularity. A study by Jürgens et al. (2010), investigated the floral scent composition of different *Asclepiadaceae* species using Micro SPE. Micro SPE coupled with (GC-MS) facilitated the detection of 151 compounds of which 103 were identified (Jürgens et al., 2010). A study carried out by Van de Niet et al. (2010) investigated the scent chemistry of several South African Orchid species on the population level. In another study carried out by Jürgens et al. (2012), the floral scent of four Hawaiian *Schieda* species was investigated via Micro SPE to detect volatiles of low molecular weight.

There are certain factors that may influence the effectiveness of SPME and Micro SPE. The columns used in the gas chromatography largely influences the resolution of the chromatographic separation. The selections of columns are generally based on the following factors: stationary phase, column internal diameter, film thickness and column length (Jennings et al., 1997). Firstly, the stationary phase refers to the coating on the inside of the column, when the sample is injected, analytes interact differently in the column; one of the compounds is generally retained for a longer time (Jennings et al., 1997). The polarity of the phase also plays a role for the selection of the best suited chromatography column. A non-polar column is best for non-polar compounds and polar column best separates polar compounds (Jennings et al., 1997). The bonding of a phase also influences the outcomes of the analyses; bonded phases are crosslinked with tubing, which exhibit less bleed and can be exposed to high temperatures, whereas a non-bonded phase comprise of a coating on the wall (Jennings et al., 1997). Secondly, the internal diameter of a column facilitates more efficient sample capacity, hence if the column has a large internal diameter then it can deal with analytes in a sample

that have a relatively large mass (Jennings et al., 1997). Thirdly, the film thickness on a column influences the resolution; decreasing thickness results in sharper peaks and reduced column bleed (Jennings et al., 1997). Lastly, the length of the column has an effect on the resolution, the length of column selected greatly depends on the application. However, most laboratories use 30 m columns because they provide the best balance between the separation of compounds and the length of analysis run (Jennings et al., 1997). In the present study a relatively non-polar column (DB5) and polar column (Carbowax) were used.

Advantages and disadvantages of SPME and Micro SPE

The advantages when using SPME are: the method is easy to use, quick and very consistent (Hyötyläinen, 2009). The fiber is conditioned in the injector port during the GC run and is then ready to use again after the analysis run is finished. Disadvantages are that the fiber may become saturated early during the collection of a sample and as a result it is not a method that can be used for accurate quantitative measurements (Matich et al. 1996). Also, fibers are fragile and a common problem includes breakage of the fiber and stripping of the coatings and bending of the needle (Hyötyläinen, 2009). Furthermore, only one sample can be collected at a time unless a second field sampler is available which is expensive to purchase.

The advantages of Micro SPE are: it is easy to use, the thermodesorption trap has a larger surface area compared to SPME, the flow rate at which samples are collected can be controlled, many samples can be collected and stored in a refrigerator until analyses, the scent traps can be easily made using glass/silica wool and Carbotrap and Tenax TA (or any other adsorbent). Scent traps can be reused by washing them in acetone and or methanol and baking them in an oven. However, disadvantages include: handmade traps may not have consistent performance, there may also be bleed-through, thermal lag due to large volume of polymer and lastly, if traps are not properly cleaned, contaminants may remain in the trap and show up as artifacts in collected samples. Finally, Micro SPE requires a specialized injector and injector device (Chromatoprobe).

Applications of SPME and Micro SPE

Both SPME and Micro SPE have been used in variety of applications. Environmental applications include the determination of aromatics of clay matrices (Zhang and Pawliszyn, 1995), VOCs emitted at landfill sites (James and Stack, 1996). SPME has been used in the food industry to assess the nutritional value of food, food additives and other contaminants and for quality control of fresh and processed products (Vas and Vekey, 2004). In the food industry, SPME is used to assess the change in VOCs of food, wine and fruits during the ripening, harvest, post-harvest and production stage (Vas and Lorincz, G. 1999), this facilitates in determining if the pattern of VOCs produced changes during the processing and storage. SPME is also used to characterize different alcohol containing beverages based on their VOC composition, SPME is widely used to identify wine VOCs and most wine VOCs are from the fermentation process (Ebeler, 2001). Other applications of SPME include hair (Sporkert and Pragst, 2000) and breath analysis (Boyle et al., 2002). Micro SPE is also used to determine VOC composition of biological matrices such as plasma, serum and whole blood, urine, milk, juices, water samples, wine and beer, soil and sediment (Amirav and Dagan, 1997).

The aim of this current study was to compare SPME and Micro SPE in terms of their relative sensitivity for analysing typical floral and fermentation volatiles that are typically encountered during the fermentation process. A range of standard compounds were tested under standardized conditions with two different chromatography columns (DB5 and Carbowax). Because Micro SPE has the potential to show bleed-through, sample vials were connected to a second Micro SPE vial in series after the first one. The outcome from this study may guide researchers to select the most adequate extraction technique for their specific research questions.

2.2. Materials and methods

2.2.1. Standard preparation

Two standard mixtures were prepared for this study (see **Table S1** in the Appendix), a mixture with typical fermentation volatiles and a mixture with compounds typically emitted from flowers. The fermentation standard was composed in a way to represent volatiles like those emitted by fruits that are rotting or going through some process of fermentation (e.g. Jürgens et al., 2013).

The floral standard blend represented odours that have been reported to be emitted by freshly opened flowers (e.g. Knudsen et al., 2006). The floral standard mixture included the following nine compounds: (i) hydrocarbon ketones: acetone; (ii) hydrocarbon esters: (3*Z*)-3-hexenyl acetate; (iii) monoterpenes: α -pinene, limonene, bornyl acetate and linalool; (iv) benzenoids/ phenylpropanoids: benzaldehyde, methyl benzoate, and methyl salicylate. The standards were prepared and stored in glass vials. For the standard blend, 950 μ L of paraffin oil was used, and then an amount of 50 μ L of each compound was added to the paraffin oil, however, with exception of acetoin which was not in liquid form. For acetoin 50 mg was dissolved in the 950 μ L paraffin oil which contained the other compounds.

A total of 18 volatile compounds were used for the fermentation standard. The compounds included were: (i) hydrocarbon ketones: acetoin and 2,3-pentanedione; (ii) hydrocarbon alcohols: (*Z*)-3-hexen-1-ol, methanol, ethanol, (iii) hydrocarbon acids: hexanoic acid, butyric acid, isovaleric acid and acetic acid; (iv) hydrocarbon esters: methyl butyrate, (*Z*)-3-hexenylacetate, hexyl butyrate, ethyl hexanoate and benzyl benzoate; (v) hydrocarbon aldehydes: octanal; and (vi) sesquiterpene: (*E*)-caryophyllene; and (vii) the benzenoids/phenylpropanoids: 2-phenylethanol, and benzyl alcohol.

2.2.2. Sample collection - SPME

The collection of samples for the floral standard involved, placing a piece of Whatman filter paper into a plastic vial and adding 1 μ L of the fermentation standard on the filter paper, the vial was instantly closed. Thereafter, the vial was left for duration of five minutes, after the waiting period; the plastic vial was placed into a 188mm x 185mm Nalophan® bags (Kalle GmbH, Wiesbaden, Germany). The bag was sealed on both ends using a heat sealer. The bag which contained the plastic vial was left for a period of five minutes. After five minutes, the plastic vial was opened in the Nalophan® bag and allowed to calibrate for a minute. Thereafter, an incision was made and a SPME holder which had a PDMS red (non-bonded) fiber was exposed to the scent compounds in the oven bag for two minutes (**Figure 1 F-H**). The standards were relatively strong and small amounts had to be added to the filter paper, as a result the time periods used in this study was based on the strength of the standard. PDMS is a thermally stable coating that can withstand temperatures ranging from 300 °C to 320°C and it has a high affinity for polar molecules (Carasek and Pawliszyn, 2006, Mallouchos et al., 2002, Pawliszyn,

2012). For the fermentation standard 10 samples were collected in total.. A control for each sampling day was taken.

2.2.3. Sample collection - Micro SPE

The Micro SPE scent traps which were used to collect the samples were conditioned in the laboratory prior to the collection of VOCs by washing them with acetone and methanol and heating them at 220°C in an oven. The scent traps used for this study were filled with 1 mg of Carbotrap® and 1 mg of Tenax® with plugs of silanised quartz wool. Samples were collected from the floral and fermentation standard to determine the sensitivity of Micro SPE and also to assess bleed-through. Bleed-through is the successive attachment of traps and release of compounds, the trap acts as a column and therefore bleed-through is also dependent on time. The collection of samples for the fermentation standard involved, placing a piece of Whatman filter paper into a plastic vial and adding 1 µL of the fermentation standard (diluted in paraffin oil) onto the filter paper - the vial was instantly closed. Thereafter, the vial was left for five minutes so that the scent could emit from the filter paper. Following the waiting period, the closed plastic vial was placed into a 188mm x185mm Nalophan® bag (Kalle GmbH, Wiesbaden, Germany). After five minutes the plastic vial was opened in the polyacetate oven bag and allowed to calibrate for a minute. A scent trap consisting of a micro vial filled with 1 mg of Carbotrap® and 1 mg of Tenax® filled with plugs of silanised quartz wool was attached to the micro-air sampler. To assess the levels of bleed-through a second scent trap was added at tandem after the first trap (connected with the same tubing used for the main sample). Bleed-through was tested for, since in strong samples this might affect whether quantitative estimates are correct. Air from these bags was then pumped through the trap with a micro-air sampler (Supelco PAS-500); the micro-air sampler was connected to an air flow meter which was calibrated at a scale of 35 which was equivalent to a flow rate of 200 mL/min (**Figure 1 A-E**). Hence, samples were collected for two minutes at a flow rate of 200 mL/min. To avoid any contamination, once the sample was collected, the scent traps were removed using an oven bag and placed in a glass vials. A total of 20 odour samples for the fermentation standard and 20 samples assessing bleed-through were collected. Samples for the floral standard were collected in the same manner. A control for each sampling day was collected from the surrounding air in order to distinguish between compounds emitted from samples and ambient compounds.

Samples were prepared and collected in the same manner for both SPME and Micro SPE to make them comparable.

2.2.4. Analyses of samples using GC-MS

Samples collected using Micro SPE were stored in a refrigerator at -20°C before analysis. GC-MS analyses of the samples were carried out using a Varian CP-3800 GC (Varian, Palo Alto, California) with a 30 m x 0.25 mm internal diameter (film thickness 0.25 µm) DB 5 column and a Carbowax column (30 m x 0.25 mm internal diameter, film thickness 0.25 µm), which was coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70eV (Shuttleworth and Johnson, 2010). For analyses, scent traps were placed in a Varian 1079 injector equipped with a 'Chromatoprobe' thermal desorption device (Amirav and Dagan, 1997). Other settings of the GC-MS were according to Shuttleworth and Johnson (2010). To quantify emission rates, known amounts of authentic standards were used. Ten of the fermentation standard and ten bleed-through samples were analysed using a DB5 column and another ten were analysed using a Carbowax column. Ten of each of the floral standard and bleed-through samples were analysed using a DB5 column and another ten were analysed using a Carbowax column. Samples were injected at 40°C in order to protect the column from the introduction of heated oxygen. For SPME, once a single sample was collected, it was instantly injected into the GC, and samples were analysed using a DB 5 column. Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011, MS search software version 2.0 d). Identification of compounds were verified using the retention times of authentic standards and published Kovats indices. The absolute scent emission rates were calculated using the peak surface area of a known amount of methyl benzoate as a reference, hence one nanolitre of methyl benzoate was injected into a thermodesorption cartridge and this was run in the GC-MS, this was repeated three times and an average of the repeats were calculated to determine the average total ion count per nanogram of methyl benzoate.

2.2.5. Statistical analyses

The difference in compounds emitted per sample were determined using multivariate statistical techniques in the software program PRIMER version 6 (Primer E). The differences in VOC composition of each sample was assessed using percentages of individual compounds. To calculate the absolute amounts the total ion counts were determined from the area under the peaks. Thereafter the absolute amounts were converted to percentages. Before calculating Bray-Curtis similarities percentage

data for compounds were square-root transformed in order to reduce the influence of outliers on the results. To visualize the data a two-dimensional representation of the data for the DB5 and Carbowax column was calculated using Non-Metric Multidimensional Scaling (NMDS). The stress value is reported to evaluate how well the particular configuration produces the observed distance matrix. Smaller stress values indicate a better fit of the produced ordination to the observed distance matrix (Clarke and Gorley 2001). In addition, to test the significance of differences between compounds produced from the fresh to wilted stage, analysis of similarities (ANOSIM) was used with 10,000 random permutations based on the pair-wise Bray-Curtis similarities between samples (Clarke and Gorley, 2001). Spearman Rank correlation was calculated for the bleed-through samples.

2.3. Results

2.3.1. Composition of SPME and Micro SPE samples

Floral scent standard mixture – The following compounds were identified in samples of the floral standard mixture when using SPME: acetone, α -pinene, benzaldehyde, (3Z)-3-hexenyl acetate, limonene, methyl benzoate, bornyl acetate and methyl salicylate (**Figure 2A**). The same set of compounds except acetone, were detected and identified when samples were collected using Micro SPE and analysed on a DB 5 column (**Figure 2B**). The compounds that were detected and identified in the samples that were collected using Micro SPE and run on a Carbowax column were the same as those with SPME, however, some compounds showed later retention times (**Figure 2C**). A significant difference was found when comparing the samples analysed on the DB5 and the Carbowax column (ANOSIM global $R = 0.164$, $P < 0.05$, **Figure 4**). For the floral standard, there was a significant difference in the absolute emission of compounds when using SPME and Micro SPE (DB5 and Carbowax column), (ANOSIM global $R = 0.688$, $P < 0.01$, **Figure 6**).

Fermentation standard mixture – The following compounds were detected and identified in the fermentation standard when using SPME as sampling method: acetic acid, 2,3-pentanedione, methyl butyrate, butyric acid, (Z)-3-hexen-1-ol, isovaleric acid, hexanoic acid, ethyl hexanoate, (Z)-3-hexenyl acetate, benzyl alcohol, 2-phenylethanol, hexyl butyrate, and (*E*)-caryophyllene (**Figure 3A**). The same set of compounds were detected and identified when samples were collected using Micro SPE analysed on a DB 5 column (**Figure 3B**). However, when using a Carbowax column differences were evident:

2,3-pentanedione, ethyl hexanoate, hexyl butyrate, and (*E*)-caryophyllene were not detected, whereas, an additional compound (i.e. octanal) was detected (**Figure 3C**). A significant difference was found when comparing the samples analysed on the DB5 and the Carbowax column (ANOSIM global $R = 0.206$, $P < 0.05$, **Figure 5**). For the fermentation standard, there was a significant difference in the absolute emission of compounds when using SPME and Micro SPE (DB5 and Carbowax column) ($P < 0.05$, **Figure 7**).

2.3.2. Bleed-through of Micro SPE samples

Floral scent standard mixture – The absolute bleed-through for the Carbowax column and DB5 column are shown in **Table 1** and **Table 2**. No significant differences were found between the absolute amounts (ng) of floral scent standard compounds in the first sample and the bleed-through detected in the second sample ($P = 0.226$, **Figure 8**). In some samples the amount detected in the bleed-through sample was even higher than that of the first Micro SPE trap, whereas in other instances no bleed at all occurred in the second trap (**Figure 8**).

Fermentation standard mixture – The absolute bleed-through for the Carbowax column and DB5 column are shown in **Table 3** and **Table 4**. There was a significant difference in the amount (in ng) of fermentation scent standard compounds found in the first sample and the bleed-through detected in the second sample ($P < 0.05$, **Figure 9**).

2.4. Discussion

2.4.1. Variation in the emission of VOCs from the floral and fermentation standard associated with the use of SPME and Micro SPE

The results from this study indicate that both SPME and Micro SPE are effective methods for the investigation of floral- and fermentation volatiles. SPME resulted in the detection of eight compounds of the floral standard (in total nine compounds), Micro SPE, when used in conjunction with a DB5 column resulted in the detection of seven compounds; however when using a Carbowax column all nine compounds were detected. For the fermentation standard (in total 18 compounds) the following

were detected: (i) SPME, 13 compounds; (ii) Micro SPE/DB5 column, 12 compounds; and (iii) Micro SPE/Carbowax column, 12 compounds.

There was a high variability between Micro SPE samples. In some instances the second trap (bleed-through) would contain higher absolute amounts than the first trap. It is possible that the high variability between Micro SPE samples are linked to bleed-through. Bleed-through is likely the result of scent traps that are not densely packed with adsorbent. In such traps the scent will not be adsorbed by the first trap but it will travel through the adsorbent and then be trapped in the second trap. Bleed-through was more evident among low molecular weight compounds. Bleed-through could be dealt with in the future by conducting control experiments before using the thermodesorption traps; e.g. a defined amount of standard and a second trap (connected in sequence to the first) are used to test the amount of bleed-through. The same should be done if traps have been used for a longer time because washing and drying them in an oven might also affect the density of the adsorbent in the trap.

The compounds that were detected when using SPME were consistent. However, it has been shown that SPME seems to be better for the absorption of high molecular weight compounds because less volatile and low molecular weight VOCs evaporate and are minimal in the headspace due to the rapid absorption of the SPME fiber (Matich et al., 1996). Lighter molecules such as ethanol are also trapped better when using SPME. Furthermore, higher molecular weight VOCs take a longer time to equilibrate as compared with the low molecular weight VOCs that equilibrate quicker (Matich et al., 1996). According to Matich et al., (1996), SPME is ideal for rapid qualitative determination of VOCs, but is limited when it comes to the quantification of complex mixtures of compounds with different molecular weights and polarity. This may also help to explain the results of the present study and the selective absorption of certain compounds. The use of SPME facilitated the detection of eight out of the nine compounds that were used to make up the floral standard mixture. The detected compounds included: acetone (58 = molecular weight), (3Z)-3-hexenyl acetate (142), α -pinene (136), limonene (136), bornyl acetate (196), benzaldehyde (106), methyl benzoate (136) and methyl salicylate (152). All the compounds that were detected had a relatively high molecular weight with exception to acetone. However, acetone was probably detected because the sampling time was two minutes, and this was long enough that this compound could be absorbed by the fiber.

For the fermentation standard, SPME facilitated the detection of 13 compounds out of the 18 that were used to create the standard. The compounds detected included: acetic acid (60 = molecular

weight), 2,3-pentanedione (100), methyl butyrate (102), butyric acid (88), (*Z*)-3-hexen-1-ol (100), isovaleric acid (102), hexanoic acid (116), ethyl hexanoate (144), (*Z*)-3-hexenyl acetate (142), benzyl alcohol (108), 2-phenylethanol (122), hexyl butyrate (172), and (*E*)-caryophyllene (204). The majority of the compounds that were used to create the fermentation standard had a relatively high molecular weight and this could have resulted in the easy absorption often, with large peak areas. SPME is better suited for qualitative analyses than precise quantitative analyses.

The use of Micro SPE facilitated easier quantification of the results. With Micro SPE, two different columns were used, DB5 and Carbowax. The results indicated that a Carbowax column was better for the detection of fermentation/rotting fruit volatiles.

2.4.2. The efficiency, reproducibility and sensitivity of SPME and Micro SPE that guide decision making

Extraction techniques play a vital role for the effective collection of VOCs. In this study, SPME and Micro SPE were compared to explore their efficiency, reproducibility and sensitivity. The SPME device consists of a polymer coated fiber which resembles a syringe (Vas and Vékey, 2004). The SPME device facilitates the collection of a single sample that requires to be injected into the GC-MS. A SPME field sampler allows the collection of at most three samples at a time that need to be injected into the GC-MS upon returning to the laboratory. Micro SPE is an active sampling method that uses a battery operated micro-air sampler (scent pump) to pump the scent accumulated air through an absorbent filled cartridge (scent trap). The scent traps that are used for Micro SPE can easily be made using silica wool, Tenax and Carbotrap and they can be reused. However, the micro-air samplers (scent pumps) are an expensive factor when considering to use Micro SPE. In terms of efficiency and handling of the methods, both SPME and Micro SPE have their advantages and disadvantages and this depends largely on the type of study. For instance, if a study requires the collection of a few samples in close proximity to a laboratory where the samples are analysed via GC-MS, SPME would be a good method since the collected samples can be immediately injected into the GC-MS. If a study entails the collection of flower VOCs that need to be collected during a field trip, Micro SPE would be more appropriate since scent traps can be made, packaged and taken on the trip along with several scent pumps. Furthermore, with Micro SPE large numbers of samples can be collected and stored in glass vials and then refrigerated and analysed at a later time.

In terms of reproducibility the results of this study indicate that SPME produces very consistent qualitative results. However, I did not vary the equilibration times and the effect of equilibration times could therefore not be determined. There was little background noise (additional peaks found in control samples of the ambient air) with SPME. Background noise was higher with Micro SPE probably because a much larger volume of air was sampled.

Sensitivity of a method is vital in facilitating the absorption of VOCs. SPME is a very sensitive method due to different fibers that are available with different coatings, each developed for a range of target chemicals. Micro SPE is quite flexible with regard to sensitivity because the air flow of the pump can be adjusted so that the method can deal with extremely weak or strong samples. When making a decision it is important to know about the limitations of each method. For SPME, the fibers tend to become saturated and as a result only a certain amount of VOCs can be absorbed. The main problem of Micro SPE is that bleed-through of compounds can occur because not all VOCs are absorbed by the trap and may pass through if the adsorbent is not packed densely enough. However, Micro SPE is easy to handle, easy to use, and many samples can be collected in a relatively short time and then stored for later analyses. Another important factor to consider is that Micro SPE can only be used if the GC is equipped with a Chromatoprobe device. Finally, there has been a debate whether SPME can be used for quantitative analyses. While some authors recommend to use SPME also for studies where quantification is needed (Vas and Vékey 2004) others reported that SPME is good for qualitative analyses but not for quantitative questions (Matich et al. 1996).

2.4.3. Concluding remarks and future perspectives

In conclusion, both methods have advantages and disadvantages and the use of each method should be based on the available GC-equipment of the user and more specifically the application. The results from this study can provide researchers with some insight into limitations of the two methods not only in terms of the sampling method. The data also revealed that choice of the chromatography column may influence the results in terms of sensitivity. The results of this study are also interesting for applications which afford the detection of volatiles with early retention times such as fermentation volatiles that indicate food spoilage. For standard applications in working environments where quantification is not a priority (e.g. food industry) SPME may be the method of choice because it is easy to use and sampling and analyzing of samples is often conducted in close proximity to each other.

Quantification with SPME is possible if internal standards are used (Vas and Vékey 2004). However, if numerous samples with a high variability in volatile composition and emission rate are to be sampled then Micro SPE is probably the method of choice. Micro SPE is also more practical and easier to handle when large numbers of samples (more than 50) are needed to be collected in the field.

Some additional factors which may influence sensitivity of SPME and Micro SPE were not investigated in this study but should be tested in the future. For instance, it is often not clear which of the different available SPME fiber coating types is the most suitable for a given application. It would therefore be interesting to know how much the fiber coating type may influence the sensitivity of the SPME method and also the quantification of compounds. Another factor that is critical and needs more attention is the sampling time which is particularly critical for SPME because of saturation effects and a bias toward absorption of compounds with a high molecular weight (Matich et al. 1996; Vas and Vékey 2004).

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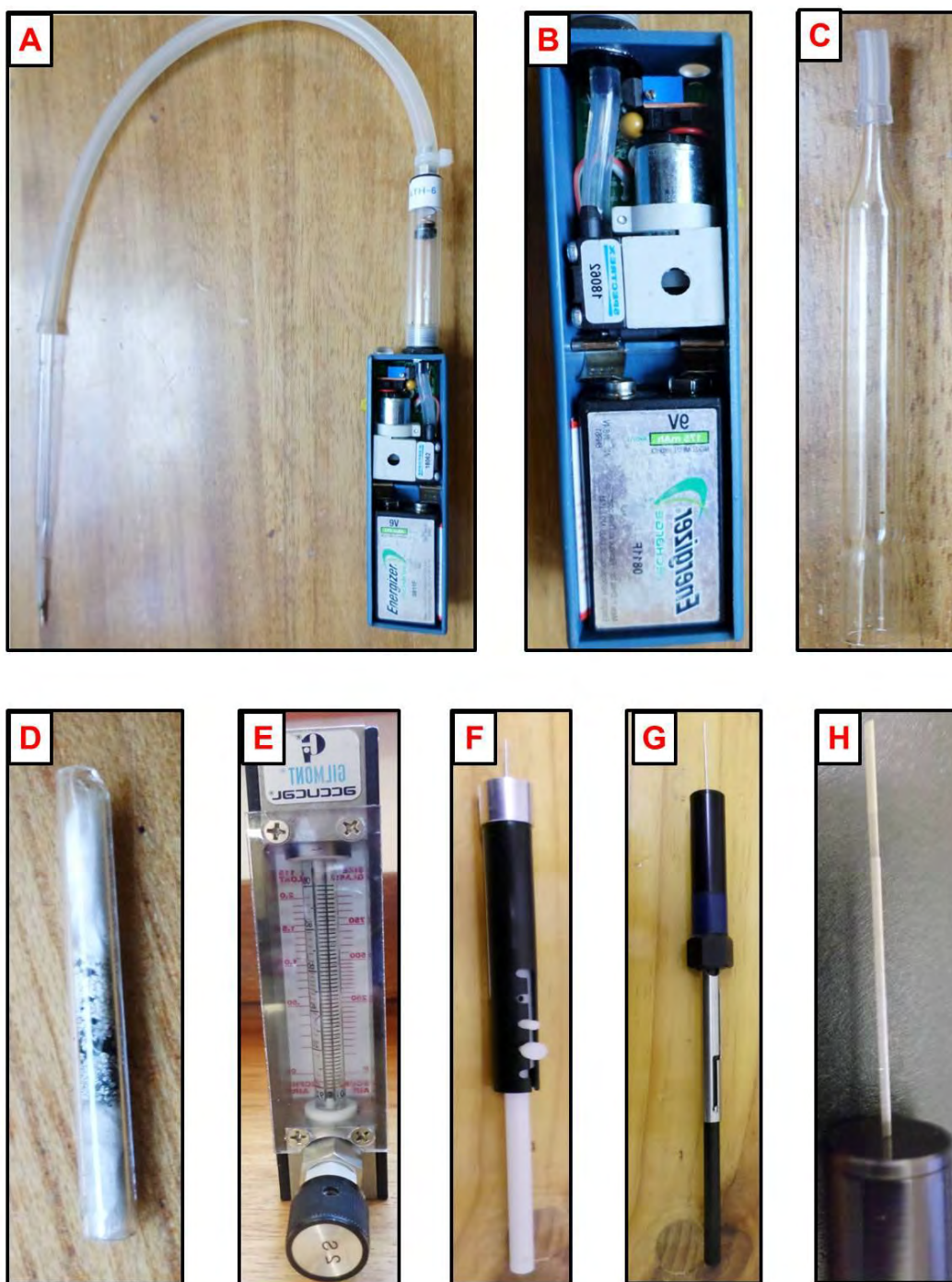


Figure 1. Equipment used for scent collection. (A) For Micro SPE a scent pump (Supelco PAS-500) was used; (B) battery and internal parts of the pump; (C) glass capillary tube that is connected to the end of the rubber tubing; (D) scent trap made of 1 mg of Carbotrap® and Tenax®; and (E) flow rate monitor which can be used to adjust the rate at which volatiles are collected. For SPME the following equipment was used: (F) a field sampler, (G) a SPME (Supelco, for details see text) holder with fiber and, (H) the fiber from a holder.

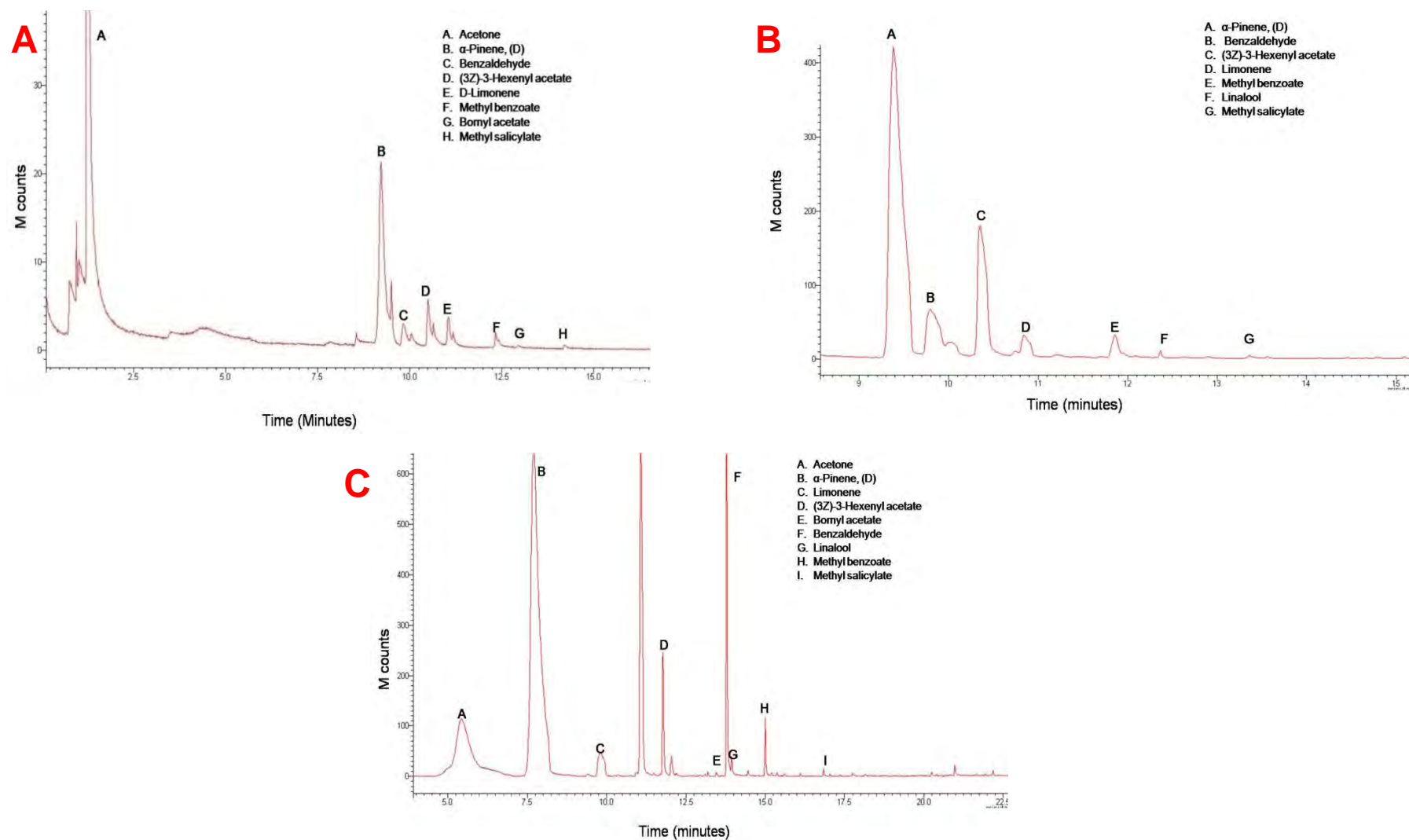


Figure 2. Compounds detected in the floral standard using (A) SPME, (B) Micro SPE (DB5 column), and (C) Micro SPE (Carbowax column)

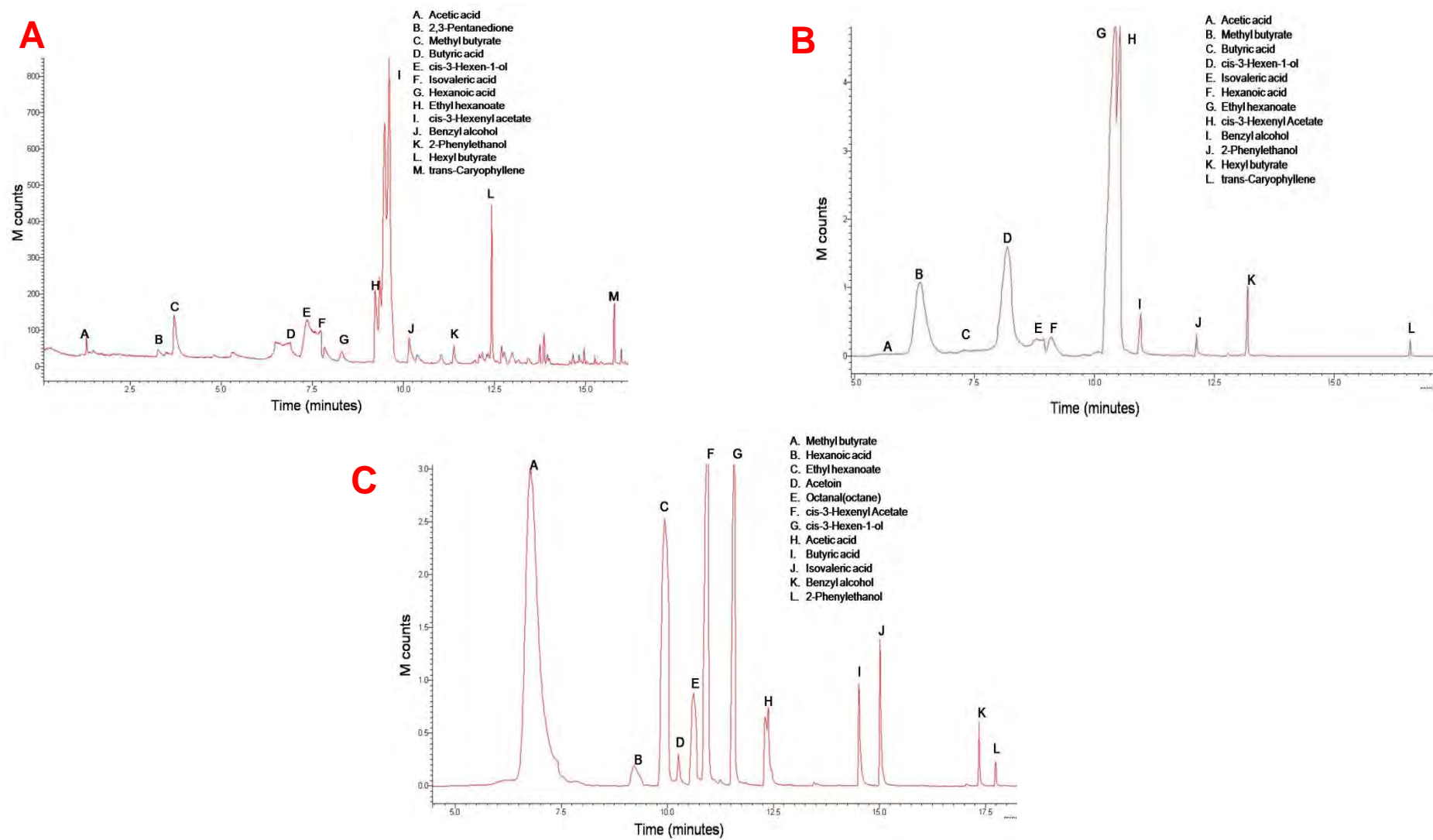


Figure 3. Compounds detected in the fermentation standard using (A) SPME, (B) Micro SPE (DB5 column), and (C) Micro SPE (Carbowax column).

floral scent

floral scent

Transform: Square root

Resemblance: S17 Bray Curtis similarity

Transform: Square root

Resemblance: S17 Bray Curtis similarity

2D Stress: 0,1

2D Stress: 0,1

column

db5

cw

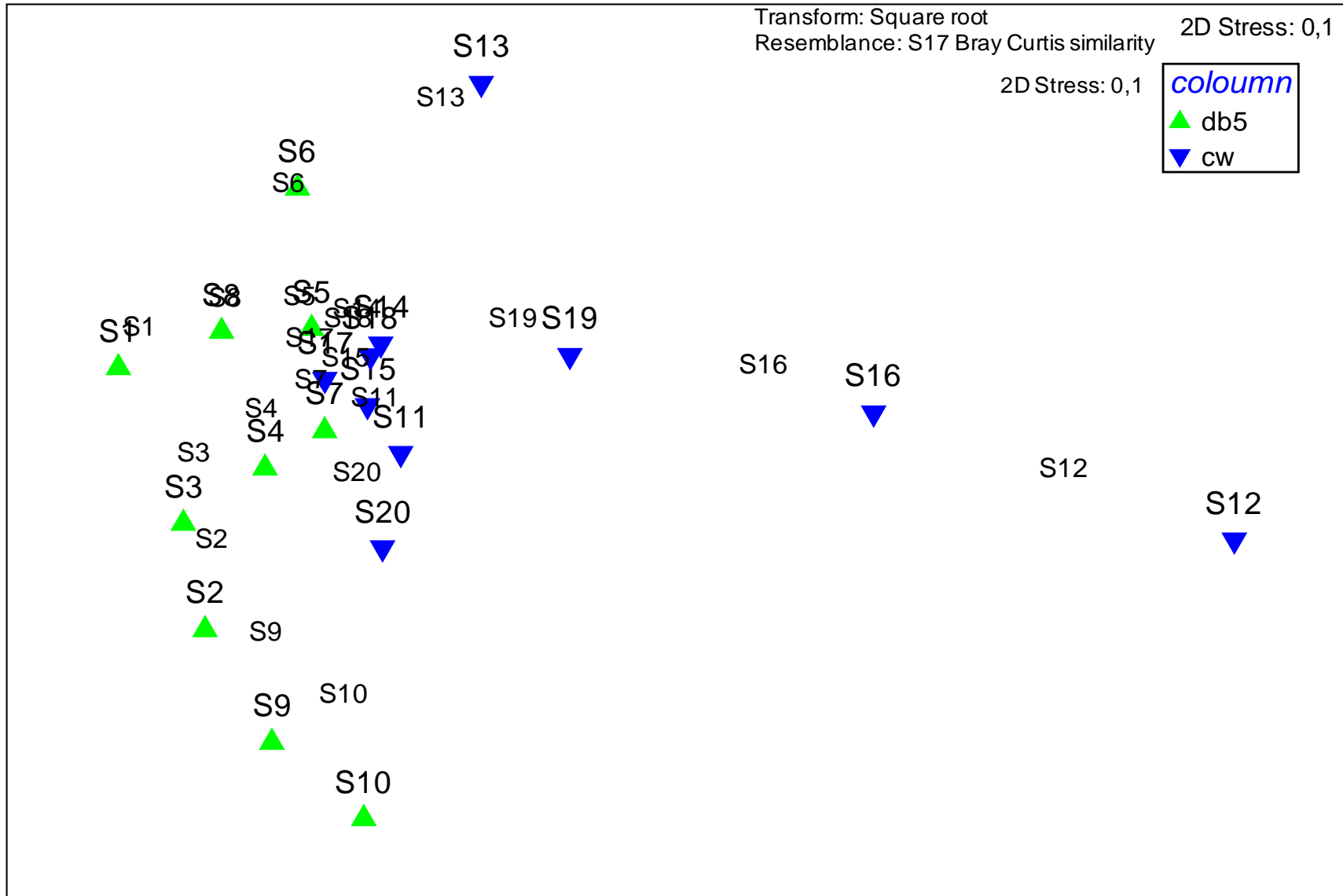


Figure 4. NMDS of floral scent standard compounds collected with thermodesorption vials and then analysed on a DB5 column (S1-10) and on a Carbowax column (S11-S20). There were significant differences. 2D stress value = 0.1, ANOSIM Global R = 0.164, $P < 0.05$).

Fermentation - Percentage

Fermentation - Percentage

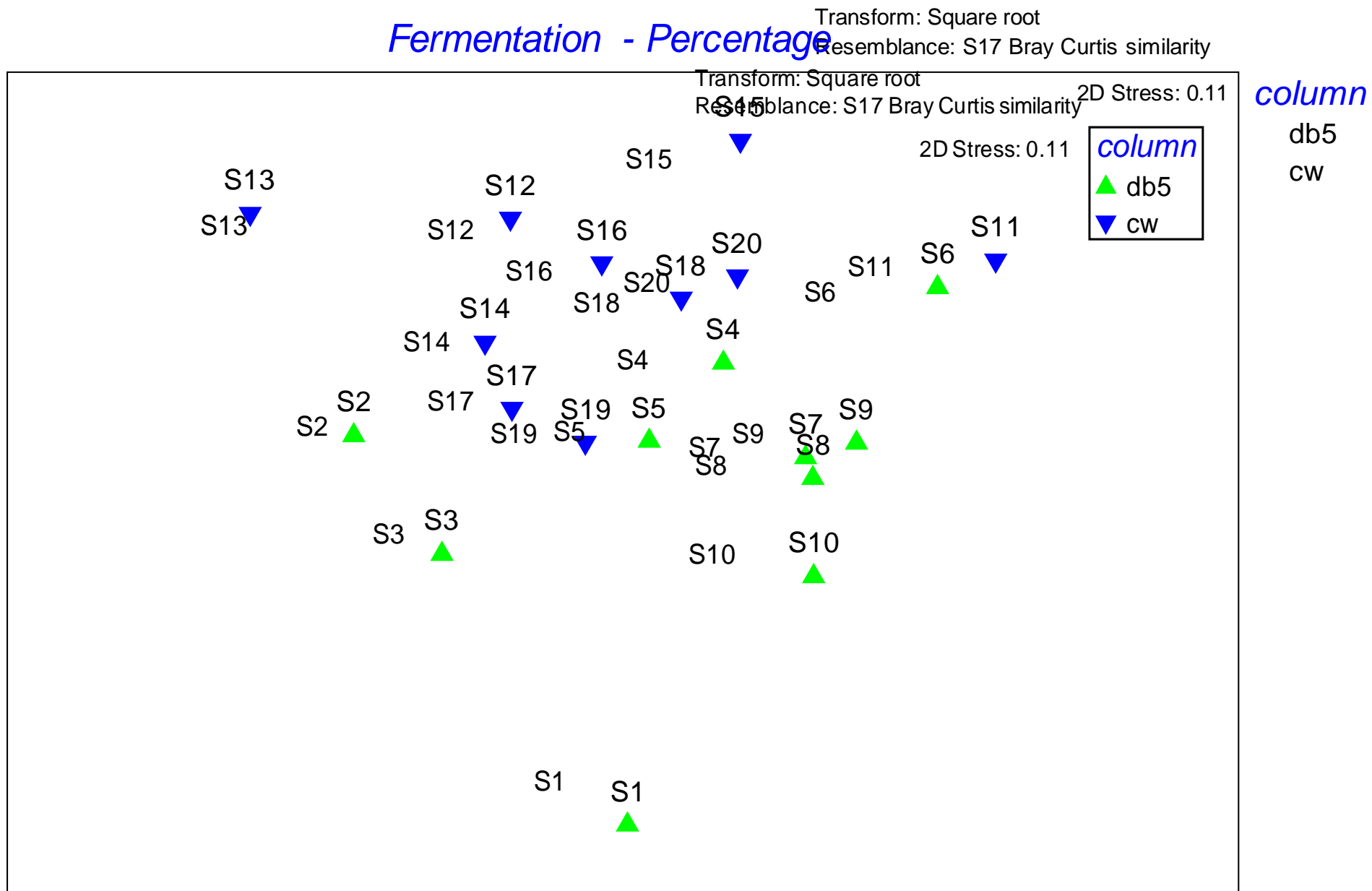


Figure 5. NMDS of fermentation scent standard compounds collected with thermodesorption vials and then analysed on a DB5 column (S1-10) and on a Carbowax column (S11-S20). ANOSIM showed that differences are significant. 2D stress value = 0.11, ANOSIM Global R = 0.206, $P < 0.05$)

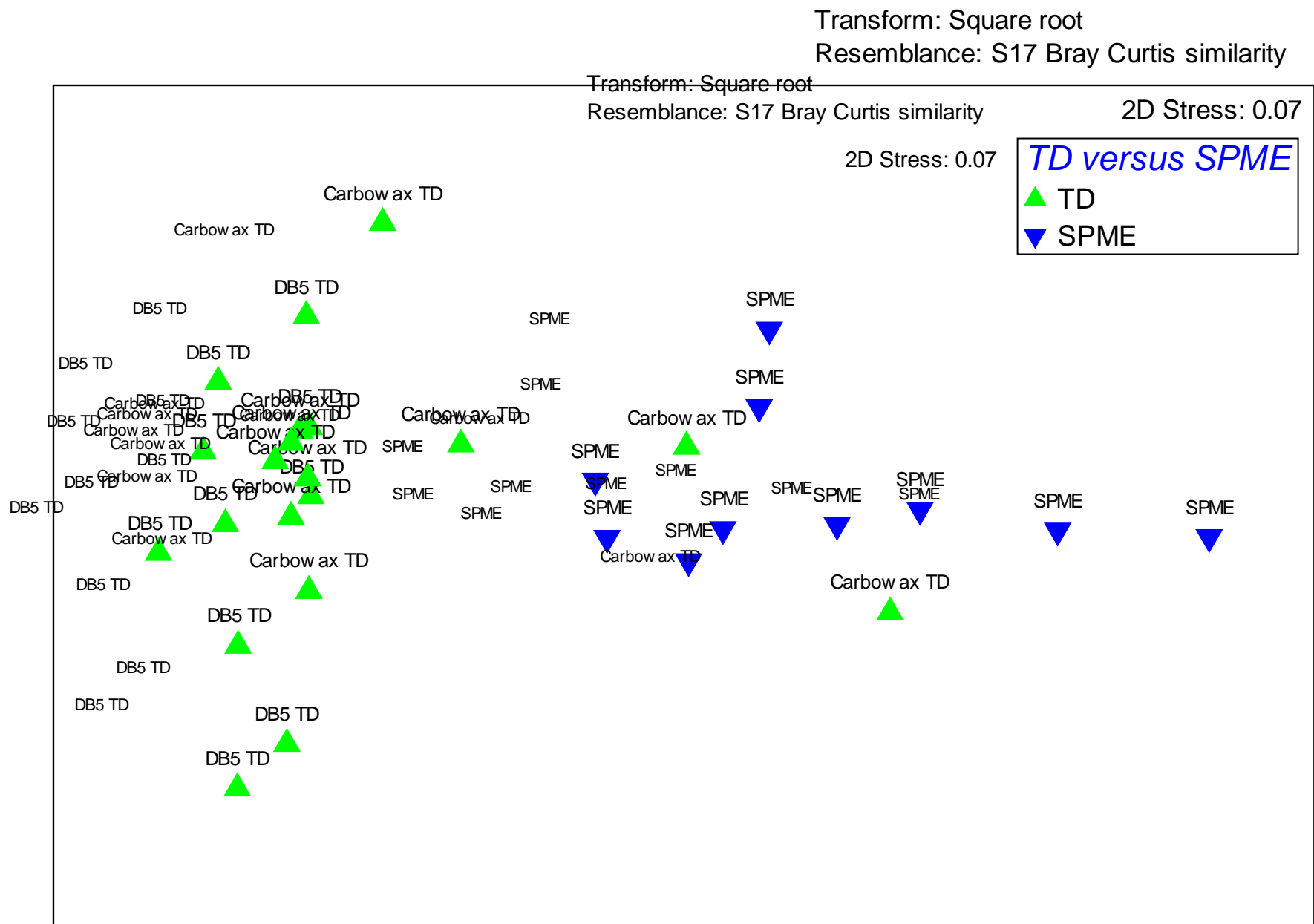


Figure 6. NMDS of floral scent standard compounds collected using Micro SPE and then analysed on a DB5 and Carbowax column and collection with SPME. ANOSIM showed that there were significant differences. 2D stress value = 0.07, ANOSIM Global R = 0.688, $P < 0.01$).

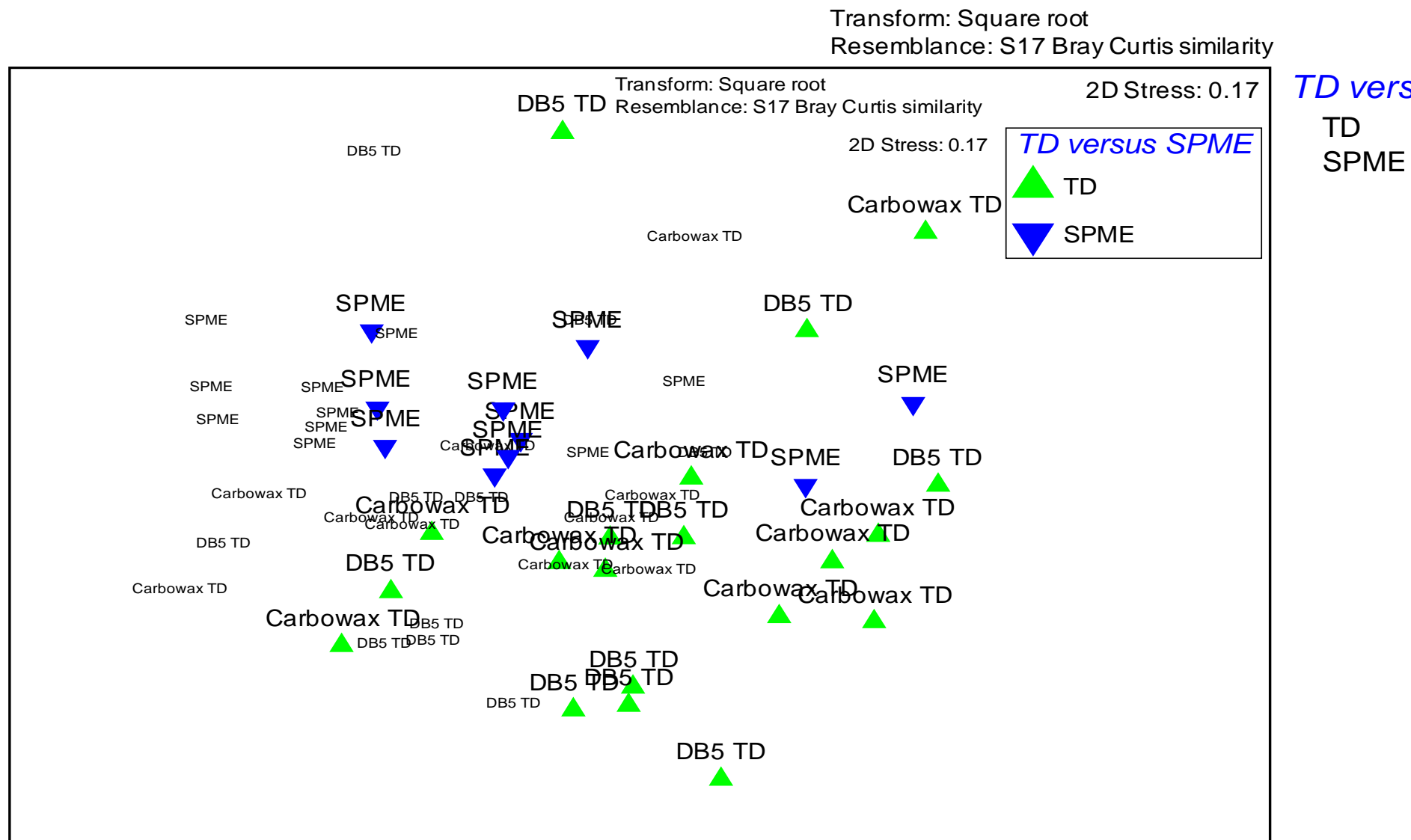


Figure 7. NMDS of the fermentation standard compounds collected using Micro SPE and then analysed on a DB5 and Carbowax column and collection with SPME. ANOSIM showed that there were significant differences. 2D stress value = 0.17, ANOSIM Global $R = 0.184$, $P < 0.05$).

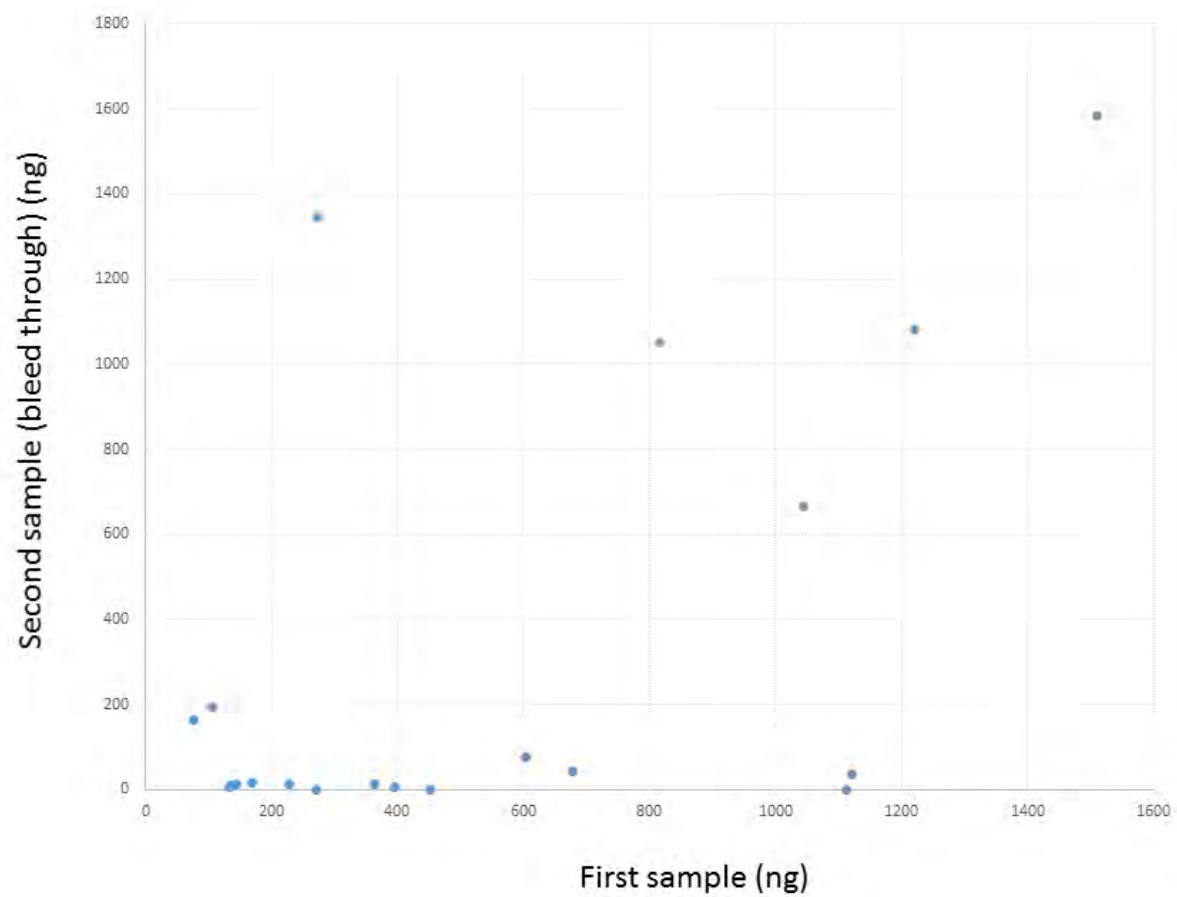


Figure 8. Relationship between the amount (in ng) of floral scent standard compounds found in the first sample and the bleed-through detected in the second sample. Spearman rank order correlation $P = 0.226$ ($n = 20$).

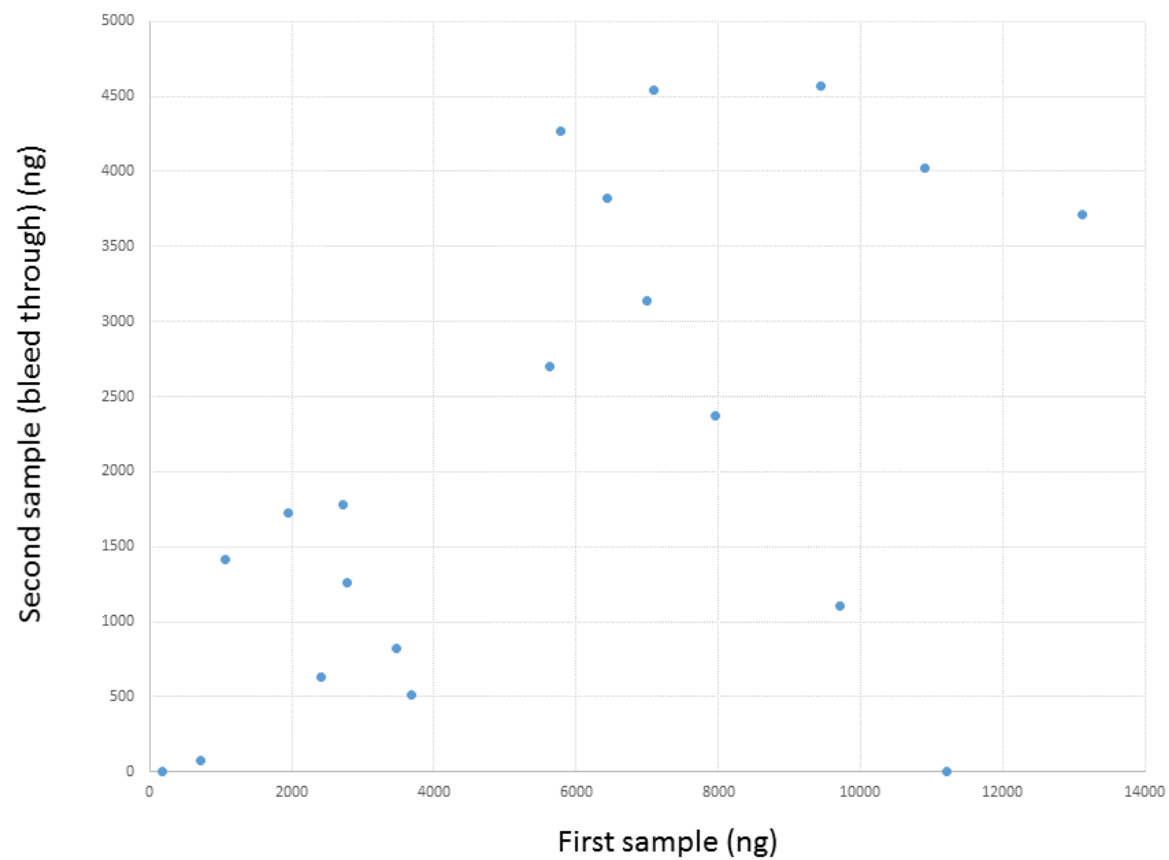


Figure 9. Relationship between the amount (in ng) of fermentation standard compounds found in the first sample and the bleed-through detected in the second sample. Spearman rank order correlation $P < 0.05$ ($n = 20$).

Table 1. Bleed-through (BT) for the floral standard (for ten samples, B1 to B10) which were analysed using a DB 5 column

Compound	BT 1	BT 2	BT 3	BT 4	BT 5	BT 6	BT 7	BT 8	BT 9	BT 10
Acetone	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
α -Pinene	96.68	0.00	0.00	100.00	100.00	18.79	97.95	88.09	100.00	94.58
Benzaldehyde	0.00	0.00	0.00	0.00	0.00	12.43	0.00	0.00	0.00	0.00
(3Z)-3-Hexenyl acetate	0.00	0.00	0.00	0.00	0.00	58.04	1.72	3.59	0.00	5.42
Limonene	3.32	0.00	0.00	0.00	0.00	10.75	0.33	8.32	0.00	0.00
Methyl salicylate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total absolute emission (ng) per sample	260	0	0	198	221	94	766	318	131	244
Total ion count bleed-through	1.64E+10	0	0	1.60E+10	5.63E+10	2.29E+10	2.14E+10	3.88E+10	6.39E+10	1.24E+10

Table 2. Bleed-through (BT) for the floral standard mixture (for ten samples, B1 to B10) which were analysed using a Carbowax column.

Compound	BT 1	BT 2	BT 3	BT 4	BT 5	BT 6	BT 7	BT 8	BT 9	BT 10
Acetone	100.00	99.58	90.60	0.00	93.41	2.67	86.15	0.00	90.01	99.04
α -Pinene	0.00	0.42	9.23	0.00	6.31	90.20	12.31	97.39	9.67	0.87
Benzaldehyde	0.00	0.00	0.08	0.00	0.09	2.75	0.55	0.94	0.15	0.03
(3Z)-3-Hexenyl acetate	0.00	0.00	0.09	0.00	0.12	4.14	0.67	1.67	0.16	0.05
Limonene	0.00	0.00	0.00	0.00	0.06	0.00	0.31	0.00	0.00	0.00
Methyl salicylate	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.00
Total absolute emission (ng) per sample	3532	19797	29013	0	19257	651	2975	1375	12228	24631
Total ion count bleed-through	2.34E+10	5.77E+10	6.92E+10	0	1.64E+10	6.99E+10	3.214E+10	1.45E+10	5.49E+10	1.21E+10

Table 3. Bleed-through (BT), total ion counts, and absolute emission for the fermentation standard (samples B1 to B10) analysed using a DB 5 column.

Compound	BT 1	BT 2	BT 3	BT 4	BT 5	BT 6	BT 7	BT 8	BT 9	BT 10
Acetic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.19	0.00
Methyl butyrate	0.00	87.64	98.83	99.05	97.99	100.00	95.35	100.00	95.59	99.36
Butyric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Acetoin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.81	0.00
(Z)-3-Hexen-1-ol	0.00	11.65	0.96	0.79	0.00	0.00	3.45	0.00	1.23	0.00
Isovaleric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Hexanoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.00
Ethyl hexanoate	0.00	0.69	0.20	0.16	2.01	0.00	1.20	0.00	0.39	0.00
Octanal	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(Z)-3-Hexenyl acetate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00
Benzyl alcohol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
2-Phenylethanol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.64
Hexyl butyrate	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(E)-Caryophyllene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total absolute emission (ng) per sample	0	15090	23105	9454	1408	11582	78133	25957	32654	57417
Total ion count bleed-through	0	1.84E+10	2.81E+10	1.15E+10	1.71E+09	2.11E+09	1.81E+09	1.27E+09	1.99E+09	1.38E+09

Table 4. Bleed-through (BT), total ion counts and absolute emission for the fermentation standard (samples B1 to B10) analysed using a Carbowax column.

Compound	BT 1	BT 2	BT 3	BT 4	BT 5	BT 6	BT 7	BT 8	BT 9	BT 10
Acetic acid	1.33	0.24	1.78	0.21	0.00	3.63	0.00	0.00	1.71	0.41
Methyl butyrate	87.00	95.93	52.78	89.43	0.00	0.00	0.00	96.65	73.35	97.56
Butyric acid	0.29	0.00	0.90	0.00	0.00	80.40	0.00	0.00	0.18	0.00
Acetoin	2.77	1.62	1.27	1.49	0.00	0.49	0.00	0.00	0.60	0.64
cis-3-Hexen-1-ol	4.06	1.09	14.43	4.12	0.00	8.16	0.00	2.43	9.08	0.59
Isovaleric acid	0.29	0.00	0.72	0.00	0.00	0.24	0.00	0.00	0.20	0.00
Hexanoic acid	0.00	0.00	0.00	0.00	0.00	2.35	0.00	0.00	3.83	0.28
Ethyl hexanoate	0.00	0.53	13.44	2.19	0.00	2.37	0.00	0.92	5.92	0.27
Octanal	0.60	0.11	2.55	0.41	0.00	0.00	0.00	0.00	0.56	0.00
cis-3-Hexenyl acetate	3.58	0.46	11.86	2.08	0.00	2.29	0.00	0.00	4.46	0.25
Benzyl alcohol	0.08	0.01	0.22	0.05	0.00	0.04	0.00	0.00	0.11	0.01
2-Phenylethanol	0.00	0.00	0.05	0.01	0.00	0.02	0.00	0.00	0.00	0.00
Hexyl butyrate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(E)-Caryophyllene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total absolute emission (ng)										
per sample	31616	69961	49493	0	43442	83111	68044	20268	83610	73727
Total ion count bleed-through	1.44E+11	1.26E+11	1.58E+11	1.77E+11	2.50E+11	1.85E+11	0	1.16E+11	1.44E+11	1.57E+11

Appendix

Table S1. Standard compounds, synonyms, CAS number, molecular formula, molecular weight, and supplier of compounds used in standard mixtures.

Compound name	Synonym	CAS Number	Molecular formula	Molecular weight	Supplier
Acetoin ≥96%	3-Hydroxy-2-butanone, Acetylmethylcarbinol	513-86-0	CH ₃ COCH(OH)CH ₃	88.11	Sigma Aldrich
2,3-Pentanedione 96%	Acetylpropionyl	600-14-6	CH ₃ CH ₂ COCOCH ₃	100.12	Sigma Aldrich
(Z)-3-hexen-1-ol ≥98%		928-96-1	C ₂ H ₅ CH=CHCH ₂ CH ₂ OH	100.16	Sigma Aldrich
Methanol ≥99%	Methyl alcohol	67-56-1	CH ₃ OH	32.04	Sigma Aldrich
Ethyl alcohol		64-17-5	CH ₃ CH ₂ OH	46.07	Sigma Aldrich
2-phenylethanol ≥99%	2-Phenylethyl alcohol, Benzyl carbinol	60-12-8	C ₆ H ₅ CH ₂ CH ₂ OH	122.16	Sigma Aldrich
Benzyl alcohol ≥99%	Benzenemethanol	100-51-6	C ₆ H ₅ CH ₂ OH	108.14	Sigma Aldrich
Hexanoic acid ≥98%	Acid C ₆ , Caproic acid	142-62-1	CH ₃ (CH ₂) ₄ COOH	116.16	Sigma Aldrich
Butyric acid ≥99%		107-92-6	CH ₃ CH ₂ CH ₂ COOH	88.11	Sigma Aldrich

Compound name	Synonym	CAS Number	Molecular formula	Molecular weight	Supplier
Isovaleric acid ≥99%	3-Methylbutanoic acid, 3-Methylbutyric acid	503-74-2	$(\text{CH}_3)_2\text{CHCH}_2\text{COOH}$	102.13	Sigma Aldrich
Acetic acid		64-19-7	$\text{CH}_3\text{CO}_2\text{H}$	60.05	Sigma Aldrich
Methyl butyrate ≥98%		623-42-7	$\text{CH}_3\text{CH}_2\text{CH}_2\text{COOCH}_3$	102.13	Sigma Aldrich
(Z)-3-hexenyl acetate ≥98%	(3Z)-3-Hexen-1-ol acetate, (3Z)-C-3-Hexenyl acetate, Leaf acetate	3681-71-8	$\text{CH}_3\text{CO}_2\text{CH}_2\text{CH}_2\text{CH}=\text{CHC}_2\text{H}_5$	142.20	Sigma Aldrich
Hexyl butyrate ≥98%		2639-63-6	$\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}_2(\text{CH}_2)_5\text{CH}_3$	172.26	Sigma Aldrich
Ethyl hexanoate ≥98%	Caproic acid ethyl ester, Ethyl caproate	123-66-0	$\text{CH}_3(\text{CH}_2)_4\text{COOC}_2\text{H}_5$	144.21	Sigma Aldrich
Benzyl benzoate ≥99%	Benzoic acid benzyl ester	212.24	$\text{C}_6\text{H}_5\text{COOCH}_2\text{C}_6\text{H}_5$	212.24	Sigma Aldrich
Octanal ≥98%		111-65-9	$\text{CH}_3(\text{CH}_2)_6\text{CH}_3$	114.23	Sigma Aldrich
(E)-Caryophyllene ≥98%	β-Caryophyllene, <i>trans</i> - (1 <i>R</i> ,9 <i>S</i>)-8-Methylene- 4,11,11-trimethylbicyclo[7.2.0]un- dec-4-ene	87-44-5	$\text{C}_{15}\text{H}_{24}$	204.35	Sigma Aldrich
Acetone ≥99%		67-64-1	CH_3COCH_3	58.08	Sigma Aldrich

Compound name	Synonym	CAS Number	Molecular formula	Molecular weight	Supplier
α -Pinene ≥98%	(1 <i>R</i> ,5 <i>R</i>)-2-Pinene, (1 <i>R</i> ,5 <i>R</i>)-2,6,6- Trimethylbicyclo[3.1.1]he pt-2-ene	7785-70-8	C ₁₀ H ₁₆	136.23	Sigma Aldrich
Benzaldehyde ≥99%	Bitter almond	100-52-7	C ₆ H ₅ CHO	106.12	Sigma Aldrich
Limonene ≥93%	(+)- <i>p</i> -Mentha-1,8-diene, (+)-Carvene, (<i>R</i>)-(+)- Limonene, (<i>R</i>)-4- Isopropenyl-1-methyl-1- cyclohexene	5989-27-5	C ₁₀ H ₁₆	136.23	Sigma Aldrich
Methyl benzoate ≥99%		93-53-8	C ₆ H ₅ COOCH ₃	136.15	Sigma Aldrich
Linalool ≥97%	(±)-3,7-Dimethyl-1,6- octadien-3-ol, (±)-3,7- Dimethyl-3-hydroxy-1,6- octadiene	78-70-6	(CH ₃) ₂ C=CHCH ₂ CH ₂ C(CH ₃)(OH)CH =CH ₂	154.25	Sigma Aldrich
Bornyl acetate ≥98%		76-49-3	C ₁₂ H ₂₀ O ₂	196.29	Sigma Aldrich
Verbenone, (L)≥93%	(1 <i>S</i> ,5 <i>S</i>)-2-Pinen-4-one, (1 <i>S</i> ,5 <i>S</i>)-4,6,6- Trimethylbicyclo[3.1.1]he pt-3-en-2-one	1196-01-6	C ₁₀ H ₁₄ O	150.22	Sigma Aldrich
Methyl salicylate	Oil of wintergreen, Wintergreen oil	119-36-8	2-(HO)C ₆ H ₄ CO ₂ CH ₃	152.15	Sigma Aldrich
α -Pinocarvone	6,6-Dimethyl-2- methylenebicyclo[3.1.1]he ptan-3-one		C ₁₀ H ₁₄ O	150.22	Sigma Aldrich

*<https://www.sigmaaldrich.com>

Chapter 3

Temporal changes in the emission of volatile organic compounds of flowers: from fresh to wilted stages

Abstract

Volatile organic compounds (VOCs) released from flowers often indicate rewards to pollinators. Flower visiting animals in turn may learn to use this information to predict the presence and quality of rewards. However, during flowering anthesis the availability of rewards can change with the age of the flower and these changes may be associated with changes in the volatiles emitted by the flower. Most research carried out thus far has primarily focused on the emission of VOCs from the fresh flowering stages to identify signals that are involved in pollinator attraction. However, VOCs emitted from older, wilted stages have so far not attracted much attention, although it is possible that they may influence the behavior of flower visitor in terms of landing on them. The aim of this study was therefore to investigate the temporal changes in floral scent emission from early flowering stages until the end of flowering including the wilted stages. For this study three plant species were selected: (1) moth-pollinated *Hymenocallis littoralis* (Amaryllidaceae), (2) bee-pollinated *Dendrobium chrysotoxum* (Orchidaceae), and (3) bee-pollinated *Camellia reticulata* (Theaceae). The floral scent of the species were collected using dynamic headspace extraction methods and then analysed via gas chromatography coupled to mass spectrometry (GC-MS). There were significant differences in the absolute emission of compounds of *H. littoralis* and of *D. chrysotoxum* from the opening to senescing stage. There was no significant difference in the absolute emission of compounds from *C. reticulata*. The data suggests that relatively fast changes in the VOC composition of flowers that senesced on the plant occurred and that these changes may potentially play a role for the interaction of flowers with flower visiting animals.

Keywords: VOCs, plant-pollinator interactions, temporal changes, floral senescence

3.1. Introduction

Flowering plants are well known for their striking diversity of floral volatile organic compounds (VOCs) that they produce (Knudsen et al., 2006). The chemical diversity of flowers has been explained, at least to some extent, to be the result of co-evolution to attract beneficial insects that facilitate pollination but also as a chemical defense mechanism against florivores (Baldwin, 2010, Das et al., 2013, Dudareva et al., 2006, Farre-Armengol et al., 2013, Yuan et al., 2009, Parachnowitsch et al., 2012). The VOCs released by flowers provide pollinators and seed dispersers with vital information such as the location, quality and abundance of rewards offered (Dudareva et al., 2006, Farre-Armengol et al., 2013, Schiestl, 2015). Floral VOCs are particularly important in flowers that bloom at night when visual cues are less accessible (Jürgens et al., 2002). When visual signals can be used by flower visitors, olfactory signals also play an important role. It has been shown that bees can detect pollen and nectar in flowers via olfactory cues (Wright and Schiestl, 2009). Volatiles enable flower visiting animals to discriminate between different plant species. According to (Chittka et al., 1999), pollinators learn VOCs emitted from species of plants and in particular those flowers that provide the most rewards and this optimizes the reward gain. Honeybees can learn odours that are not associated with a reward and they are able to avoid recently visited and nectar depleted flowers based on footprint cues left by previous visitors (Wright and Schiestl, 2009, Wright et al., 2002). Honeybees learn floral rewards associated with scent cues much faster than visual cues alone (Wright and Schiestl, 2009, Wright et al., 2002). In addition, VOC emission from flowers may influence flower constancy of pollinators which in turn contributes to effective pollen transfer, reduction in pollen loss and an increase in reproductive success (Huber et al., 2005). Finally, floral VOCs enable pollinators to discriminate between species of flowers or individuals within a species (Dudareva et al., 2006, Farre-Armengol et al., 2013).

The constituents of floral VOCs include the production and release of VOCs from the petals, sepals, pollen, and nectar of the plant (Dötterl and Jürgens, 2005, Farre-Armengol et al., 2013). In most species of flowering plants, the volatiles from the sepals and petals usually make up the scent of the entire flower (Dötterl and Jürgens, 2005, Farre-Armengol et al., 2013). VOCs produced in the sepals and petals are primarily hydrocarbons (e.g. fatty acid derived alcohols, esters, aldehydes, and ketones), benzenoids (e.g. aromatic- esters, alcohols, aldehydes)

phenylpropanoids, nitrogen-bearing compounds, and terpenoids (e.g. monoterpenes, sesquiterpenes, diterpenes) (Dötterl and Jürgens, 2005, Farre-Armengol et al., 2013, Knudsen et al., 2006). The emission of VOCs from the sepals and petals is dependent upon the age of the flower (Bergougnoux et al., 2007). Other parts of the flower which contribute to the entire flower fragrance are pollen and nectar. The VOC emission from pollen is normally relatively low but some species may emit stronger pollen odours, thereby indicating to visitors that pollen which is a protein and lipid rich source, is the reward (Dobson, 1988, Dobson and Bergström, 2000, Dobson et al., 1996).

While many studies have focused on the emission of VOCs from the fresh stages of flowers, little research has been carried out on the emission of VOCs from the senescing stages of flowers. A long lived flower may produce a certain set of VOCs during the early flowering stages to attract pollinators and then upon reaching a certain life stage, independent of whether pollination occurred, produces due to senescence a different set of volatiles. Older, senescing flowers often do not produce nectar or are depleted and flower visiting animals should avoid visiting such flowers. From an ecological perspective changes in the scent composition may influence pollinator behavior with regard to flower visitation and such behavioural changes may in turn affect the plant's pollination success.

The aims of this study were to compare: (i) the temporal change in the emission of floral VOCs from three different flowering plant species: *Hymenocallis littoralis*, *Dendrobium chrysotoxum*, and *Camellia reticulata*, from early flowering until complete wilting/withering, and (ii) to determine if the number of compounds emitted changes as the flower progresses from a fresh to wilted state.

3.2. Materials and Methods

3.2.1. Study species

Flowers located at the University of KwaZulu-Natal Botanical Garden were used for this study. Flower species used for the study included: *Hymenocallis littoralis* (Jacq.) Salisb (Amaryllidaceae), which is a bulbous perennial herb, between 60 to 70 cm in height and it has

large white flowers that are moth pollinated (**Figure 1**); flowers last for a duration of four days (personal observation). *Dendrobium chrysotoxum* Lindl. var. *delacouriigagnep* (Orchidaceae), is between 25 to 30 cm which consists of inflorescences of small flowers which are bee pollinated, flowers are between 3 to 5 cm in diameter and they last for up to 13 days (personal observation), (**Figure 2 to Figure 4**). The flowers of *Camellia reticulata* f. *simplex* Sealy (Theaceae) range from 5 to 8 cm in diameter and have many anthers that are pollinated by bees (**Figure 5**). The flowers of *C. reticulata* were collected in the fresh and wilted stage for VOC comparison between stages. These species of flowers were selected for this study due to their variation in longevity.

3.2.2. Volatile sampling

Flower volatiles were collected using dynamic headspace extraction methods for subsequent analysis with gas chromatography-mass spectrometry (GC-MS). In terms of samples size, seven fresh flowers of *H. littoralis*, 10 fresh flowers and 10 wilted flowers of *C. reticulata* and 10 inflorescences of *D. chrysotoxum* were used in the study. To accumulate volatiles, flowers/inflorescences were bagged using Nalophan® bags (Kalle GmbH, Wiesbande, Germany) for a period of 20 minutes.. A scent trap consisting of a micro vial filled with 1 mg of Carbotrap® and 1 mg of Tenax® and plugs of silanised quartz wool was then inserted into each polyacetate bag. The micro vials/scent traps that were used to collect the flower volatiles were conditioned in the laboratory prior to the collection of the fruit scent by washing them with acetone and methanol and heating them at 220°C in an oven. Air from these bags was then pumped through the trap with a micro-air sampler (Supelco PAS-500) at a flow rate of 50 ml/min for 20 minutes. Samples were stored in a freezer at -20°C until analysis. Samples from *H. littoralis* and *D. chrysotoxum* were collected daily until the flowers completely wilted. The flowers of *H. littoralis* and *D. chrysotoxum* remained attached to and wilted on the plant. The daily collection of scent indicated a variation in volatiles and a rise in fermentation volatiles upon wilting. Volatile samples were collected for four days for *H. littoralis* and 13 days for *D. chrysotoxum*. Flowers of *C. reticulata* were found during the fresh and wilted stages on the tree. Therefore, fresh and wilted flowers of *C. reticulata* were broken of the tree and then bagged and scent was collected, complete variation in volatiles from the fresh to wilted stage was

determined. Controls were taken during every sampling event, which involved the collection of scent from an empty bag. Controls facilitated the detection of ambient compounds.

In total, 28 samples were collected for *H. littoralis* and 130 for *D. chrysotoxum* during their life cycle from the fresh to the wilted stage. For *C. reticulata* 40 samples were collected, 10 samples during the fresh and 10 samples during the wilted stage.

3.2.3. Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis of floral scent samples were carried out using a Bruker 450 GC with a 30 m x 0.25 mm internal diameter (film thickness 0.25 µm) Bruker DB5 or Alltech Carbowax column (30 m x 0.25 mm internal diameter, film thickness 0.25 µm) connected to a 11 m Bruker DB1 column (in series) coupled to a Bruker 300-MS quadrupole mass spectrometer in electron-impact ionization mode at 70eV. *H. littoralis* and *D. chrysotoxum* samples were analysed using a DB5 column and *C. reticulata* on a Carbowax column. Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011, MS search software version 2.0 d). Identification of compounds were verified using the retention times of authentic standards and published Kovats indices. The absolute scent emission rates were calculated using the peak surface area of a known amount of methyl benzoate as a reference, hence one nanolitre of methyl benzoate was injected into a thermodesorption cartridge and this was run in the GC-MS, this was repeated three times and an average of the repeats were calculated to determine a the average total ion count per nanogram of methyl benzoate. Compounds present at similar abundance in the controls were considered to be contaminants and excluded from the analysis. Other settings of the GC-MS were according to Shuttleworth and Johnson (2010).

3.2.4. Statistical analyses

To determine the difference in compounds emitted per sample for the different days of sampling different multivariate statistical techniques in the software program PRIMER version 6 (Primer

E) was used. Due to the variation in VOCs emitted per individual samples the differences in VOC composition of each sample was assessed using percentages of individual compounds. The absolute amounts were derived from the total area under the peaks and thereafter the absolute amounts were converted to percentages. Percentage data for compounds were square-root transformed before calculating Bray-Curtis similarities to reduce the influence of outliers on the results. A two-dimensional representation of the data was obtained using Non-Metric Multidimensional Scaling (NMDS). To evaluate how well the particular configuration produces the observed distance matrix, the stress value is reported. According to Clarke and Gorley (2001) smaller stress values indicate a better fit of the produced ordination to the observed distance matrix. In addition to test the significance of differences between compounds produced from the fresh to wilted stage, analysis of similarities (ANOSIM) was used with 10,000 random permutations based on the pair-wise Bray-Curtis similarities between samples (Clarke and Gorley, 2001). The ANOSIM test calculates the test statistic R, assessed by random permutations of the grouping vector to obtain an empirical distribution of R under the null model, as well as a level of significance. I also assessed the extent to which individual compounds contributed to the dissimilarity of the scent of the flowers from the fresh to the wilted state by using the SIMPER function in PRIMER, with the day of sampling being the factor.

Generalized linear models were used to assess the differences in the emission and number of compounds emitted over the longevity of the flowers. A gamma distribution with log link model was used to assess the differences in the emission of compounds, with time (day) as a subject variable and an auto regressive correlation matrix structure was chosen for large data sets (*H. littoralis* and *D. chrysotoxum*) and exchangeable correlation matrix for a small data set (*C. reticulata*) and the means were back transformed. A poisson distribution with log link linear model was used to assess the difference in the number of compounds emitted, with time (day) as a subject variable, auto regressive structure was chosen for large data sets (*H. littoralis* and *D. chrysotoxum*) and exchangeable for a small data set (*C. reticulata*) and the means were back transformed.

The curve estimation function in SPSS was used to determine the best fitting regression equations. Least square regression models were fit through the means and the model that displayed the highest R^2 was regarded as the best model.

3.3. Results

3.3.1. Temporal changes of the floral scent composition in *Hymenocallis littoralis*

There was a significant difference in the emission (ng) of VOCs as *H. littoralis* progressed from a freshly open to wilted stage (ANOSIM global $R = 0.39$, $P < 0.0001$, **Figure 6**). There was a high variation in the emission of VOCs during the fourth day of sampling (**Figure 6**). There was little difference in the VOC composition of samples from the first to the third day of sampling (**Figure 6**). The ANOSIM between the sampling groups 1 and 2 and 1 and 3, although significant, showed relatively low R values ($R_{(1 \text{ versus } 2)} = 0.19$, $p < 0.05$; $R_{(1 \text{ versus } 3)} = 0.27$, $P < 0.01$).

Whereas, differences between sampling groups 1 and 4 were much higher ($R_{(1 \text{ versus } 4)} = 0.78$, $P < 0.001$). During the freshly opened stage of *H. littoralis*, the main compounds which primarily contributed to the whole flower scent include: linalool (28.5%), (1Z)-2-methylbutanal oxime (20.2%), and 2-methyl-6-methylene-1,7-octadien-3-one (10.3%). On the second day of sampling of *H. littoralis*, it was determined that the main contributing compounds to the whole flower scent included: (1Z)-2-methylbutanal oxime (21.8%), benzyl alcohol (21.27%), and linalool (11.8%). During the third day of sampling, the main compounds that contributed to the scent of *H. littoralis* were: linalool (14.6%), (1Z)-2-methylbutanal oxime (9.8%), and 1,1-dimethyl-3-methylene-2-vinylcyclohexane (7.7%). On the last day of sampling, wilted *H. littoralis* flowers were dominated by: (3E)-3-hexenyl acetate (13.43%), heptenal (12.4%), nonanal (11.7%), 7-octen-2-ol, 2,6-dimethyl- (10.0%).

There was a significant difference in the emission of compounds over the longevity of *H. littoralis* ($\chi^2 = 44.060$, $df = 3$, $P < 0.005$, **Figure 9A**). There was no significant difference in the number of compounds emitted from *H. littoralis* during the four days of its life cycle ($\chi^2 = 2.81$, $df = 3$, $P = 0.421$, **Figure 9B**).

3.3.2. Temporal changes of the floral scent composition in *Dendrobium chrysotoxum*

A total of 27 compounds were found in *D. chrysotoxum*. There was a significant difference in the overall emission (ng) of VOCs from *D. chrysotoxum* from the open to wilted stage (ANOSIM

global $R = 0.31$, $P < 0.0001$, **Figure 7**). There was no difference in the compounds emitted from the flowers of *D. chrysotoxum* during sampling days 1 to 11; however on day 12 and 13, the system experienced a drastic change in scent emission (**Figure 7**). Pair-wise tests showed that when samples were grouped, compounds varied vastly on the twelfth and thirteenth day of sampling with: $R > 0.80$, $P < 0.0001$. The main compounds that contributed to the scent of *D. chrysotoxum* from day 1 to 11 were: β -myrcene, linalool, limonene, 3,7-dimethyl-1,3,7-octatriene and α -pinene. During sampling days 12 to 13, *D. chrysotoxum* was dominated by 2,4-dimethyl-1-heptene, limonene, terpinen-4-ol and (*Z*)-verbenol.

There was a significant difference in the emission of compounds over the longevity of *D. chrysotoxum* ($R^2 = 0.128$, $\chi^2 = 117.876$, $df = 12$, $P < 0.0005$, **Figure 10A**). A quadratic function was the best fitting least squares regression model. The quadratic polynomial regression equation to describe the relationship between the time and the emission of compounds is as follows: (i) the emission of compounds: $y = 2126.07 + 7385.13x - 596.19x^2$ (**Figure 10A**), (ii) the number of compounds emitted: $y = 8.12 + 2.53x - 0.02x^2$, (**Figure 10B**). There was a significant difference in the number of compounds emitted from *D. chrysotoxum* during the 13 days of its life cycle ($R^2 = 0.331$, $\chi^2 = 117.240$, $df = 12$, $P = 0.0005$, **Figure 10B**).

3.3.3. Temporal changes of the floral scent composition in *Camellia reticulata*

A total of 27 compounds were found in scent samples of *C. reticulata*. There was a clear difference in the VOC composition between the fresh to wilted stage of *C. reticulata* flowers (ANOSIM global $R = 0.81$, $P < 0.0001$, **Figure 8**). This was confirmed by ANOSIM showing a significant difference in compounds emitted during the wilted stage (ANOSIM global $R = 0.81$, $P < 0.0001$). Using SIMPER the main compounds that contributed to the scent of *C. reticulata* during the fresh stage were: ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl) propan-2-yl carbonate (37.4%), linalool (13.3%), and 2H-pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl- (10.9%). The wilted stage of *C. reticulata* flowers were dominated by benzaldehyde, benzyl alcohol, (*E*)-2-Hexen-1-ol, and 2-Heptanol.

There was no significant difference in the emission (ng) of compounds during the open and wilted stages *C. reticulata* ($\chi^2 = 0.38$, $df = 1$, $P = 0.534$, **Figure 11A**). However, there was a significant difference in the number of compounds emitted from *C. reticulata* during the open and wilted stages ($\chi^2 = 7.60$, $df = 1$, $P = 0.006$, **Figure 11B**).

3.3.4. Observation of the process of wilting in *H. littoralis*, *D. chrysotoxum* and *C. reticulata*

The corolla of *H. littoralis* began to dry out, the 8 – 10 cm stigma and anthers also dry out and sag (**Figure 1 E-F**). The flowers on the inflorescence of *D. chrysotoxum* gradually began to wilt on day 10, followed by complete wilting of flowers on day 13 (**Figure 3 J-L**), (**Figure 4M**). The flowers of *C. reticulata* were collected during the fresh and wilted stages, the flowers gradually dried on the tree of *C. reticulata* (**Figure 5F**).

3.4. Discussion

3.4.1. Temporal variation in floral VOCs

The life cycle of a typical flower goes through a bud stage, a flowering stage followed by wilting or withering of the flower or floral parts (Kotze et al., 2010). Three main types of wilting can be distinguished in flowers: (a) the first type shows a slow change in the state of the corolla and loss of turgor that is then accompanied by final wilting, (b) in the second type the life of the corolla is ended by abscission, and (c) in the third type plants drop flowers that look relatively fresh (Halevy, 1986). Based on the above types of wilting, all three investigated species, i.e. *H. littoralis*, *D. chrysotoxum* and *C. reticulata* falls under category “a” of wilting, hence the flowers displayed a slow change in the state of the corolla and loss of turgor and then final wilting. In addition, each of the types of wilting may be linked to a varied VOC emissions, since wilting is a form of physiological breakdown of tissues and this may also have consequences for VOC emission. The physiological breakdown during wilting may also allow bacteria and fungi to feed on plant tissue because of a breakdown of defense mechanisms against microorganisms.

The primary composition of VOCs released from the fresh stages of the *H. littoralis* were alcohols, whereas, the wilted stage contained aldehydes, and esters. The flowers of *H. littoralis* displayed a gradual temporal change in the VOC composition (**Figure 6**). During the fresh stage of *D. chrysotoxum* flowers, between five to 6 compounds were found in the samples, whereas during the wilted stages only three compounds were found. Limonene was emitted until day 12 and no traces were found on the thirteenth day. An abrupt change in VOC emission was found in *D. chrysotoxum* flowers (**Figure 7**). *C. reticulata* flowers were observed during their fresh and wilted stage, however a clear difference in the emission of VOCs from the fresh to wilted stage can be seen (**Figure 8**). Linalool dominated during the fresh and wilted stage of the flowers.

Typical green-leaf volatiles produced during the early stages from *H. littoralis*, *D. chrysotoxum* and *C. reticulata* included: (Z)-3-hexenyl acetate, (Z)-3-hexen-1-ol, hexanal and (Z)-3-hexenal (Pichersky and Gershenzon, 2002). VOCs emitted from fresh flowers of *H. littoralis*, *D. chrysotoxum* and *C. reticulata* included: myrcene, (3E,6E)- α -farnesene and methyl benzoate (Dudareva et al., 2000). Fermentation volatiles released from the flowers included: hexyl acetate, isobornyl formate, acetic acid and 2,3-butanediol, (Mateo et al., 2001).

Overall, linalool was a common VOC which was produced and released by all three species of flowers. Linalool is one of the most common scent compounds emitted from flowers (Parachnowitsch et al., 2012). Linalool was emitted from the fresh to the wilted stages of the flowers. Linalool has two isomers, in a study carried out using *Penstemon digitalis*, it was found to produce (S)-(+)-linalool rather than (R)-(-)-linalool, and linalool was found to be the target VOC which attracted pollinators (Parachnowitsch et al., 2012). Insects may be using the linalool to detect nectar rewarding flowers (Parachnowitsch et al., 2012). However, linalool like other monoterpenoids may serve as a deterrent to not beneficial flower visitors (Parachnowitsch et al., 2012). Linalool is a polar molecule and may be absorbed into the nectar of *P. digitalis*, thereby signaling the presence of nectar in flowers (Parachnowitsch et al., 2012). In relation to this study, all three species released linalool, therefore it could be inferred it has antimicrobial properties with respect to the presence of yeasts and bacteria in flower nectar, however further studies would be required to validate this statement.

3.4.2. Why do flowers undergo temporal changes in VOCs?

The main role of floral VOCs is to attract pollinators to flowers. However, while these VOCs attract mutualistic partners, it may also attract antagonistically interacting organisms that may negatively influence the fitness of the plants (Schiestl, 2015). Antagonistically interacting organisms include nectar robbers or inefficient pollinators which may eavesdrop on signals released by the plant to attract visitors (Schiestl, 2015). There are a few ways in which plants can overcome this problem. Firstly, plants may release VOCs that repel unfavorable visitors, together with VOCs that attract pollinators (Junker and Blüthgen, 2010, Kessler et al., 2013). Secondly, plants could restrict the emission of VOCs to times of the day when their particular pollinators are most active (Theis et al., 2007). The change or cessation of the production and release of VOCs post-pollination has two benefits: resources are conserved since the production and release of VOCs utilizes a large amount of energy and the cessation of VOCs from pollinated flowers will be beneficial to plants that contain inflorescences of flowers, since the unattractiveness of the pollinated flowers will guide pollinators to unpollinated flowers thereby increasing the reproductive success of the plant (Schiestl and Ayasse, 2001). Temporal changes are also important in moth pollinated plants since moths are active between dusk and dawn.

H. littoralis, *D. chrysotoxum* and *C. reticulata* displayed temporal variation in the emission of VOCs. Temporal changes in the emission of VOCs could be attributed to plants saving energy, specific compounds emitted may deter pollinators or the plant has used up resources and cannot produce VOCs. For instance, in a study carried out by Dudareva et al. (2000) on Snapdragon flowers, three compounds, myrcene, (*E*)-ocimene and methyl benzoate were reported to be the main compounds emitted. Methyl benzoate was produced in the largest amounts from the flowers. However, the emission of methyl benzoate was regulated by the amount of benzoic acid (the substrate) and carboxyl methyl transferase (BAMT) an enzyme present in the flowers (Dudareva et al., 2000). Methyl benzoate was not produced during the bud stages of the flowers, the emission of methyl benzoate began at anthesis and reached a peak from day 5 to 8 and thereafter declined (Dudareva et al., 2000). The emission of methyl benzoate was at its peak from 9 am until 5 pm during increased pollinator activity, emission was lower during night (Dudareva et al., 2000). It was also found that the upper and lower petal lobes were the primary source of VOC emission (Dudareva et al., 2000). Younger snapdragon flowers emitted

fewer VOCs and were less attractive to pollinators due to the anthers which were not fully developed (Dudareva et al., 2000). During the senescing stage of the flowers the overall emission of methyl benzoate declined, the BAMT activity remained high however the limiting factor was the availability of the benzoic acid which is vital for the emission of methyl benzoate (Dudareva et al., 2000).

3.4.3. The temporal change in flower VOCs post-pollination

As earlier stated, pollinators are attracted using VOCs. Post-pollination changes include: withering, closing or abscission of petals, colour change, positional adjustment (flowers become inaccessible to pollinators), cessation of nectar production and scent alteration (Theis and Raguso, 2005). The production and release of VOCs will decline once the flower has been pollinated and it senesces. It has been suggested that this will reduce the attraction of herbivores, prevent ineffective pollen deposition, and reduce the metabolic cost involved in producing the compounds (Dudareva and Pichersky, 2000). A study carried out on two thistle species, *Cirsium arvense* and *C. repandum* (Asteraceae), which are pollinated by several species of insects found that the emission of VOCs decreased in both species once pollinated and that pollinators preferred to visit unpollinated flower heads (Theis and Raguso, 2005). Furthermore unpollinated flower heads continued to emit VOCs but at a lower rate (Theis and Raguso, 2005). A similar trend was reported in *Silene latifolia*, where pollinated flowers significantly lowered their emission of scent as compared with the unpollinated flowers (Muhlemann et al., 2006).

3.4.2. Synthesis & Future perspectives

In conclusion, *H. littoralis*, *D. chrysotoxum* and *C. reticulata* displayed significant temporal changes in their VOC composition during flowering. With *H. littoralis*, more emission of compounds occurred on day one of flowering, and then decreased during day two and three. More compounds were emitted during the later wilting stages of *H. littoralis* and this could be attributed to the presence of alcohols and esters due to the wilting process. *D. chrysotoxum* had higher emissions from day one to seven and thereafter decreasing, this could be attributed to the

plant not needing to attract pollinators therefore it reduced the emission of compounds thereby reducing energetic costs. For *C. reticulata*, more emission of compounds took place during the wilted stages.

Future studies should determine the effect that the emission of early and late stage compounds have on visitors of these flowers. This can be facilitated by carrying out behavioural tests, whereby standards could be created for each flower based on the compounds that they emit (which have already been determined). The standards can then be offered to the pollinators on artificial flowers/petri dish and the attraction or repulsion can be determined. Behavioural tests will facilitate a better understanding of each of the above plant systems. Important is also to consider that the response to the compounds are very likely dosage dependent. Therefore, standard mixtures consisting of different concentrations of VOCs typically found in fermentation/wilting flowers need to be tested to show which of the late stage odours may adversely affect flower visitors.

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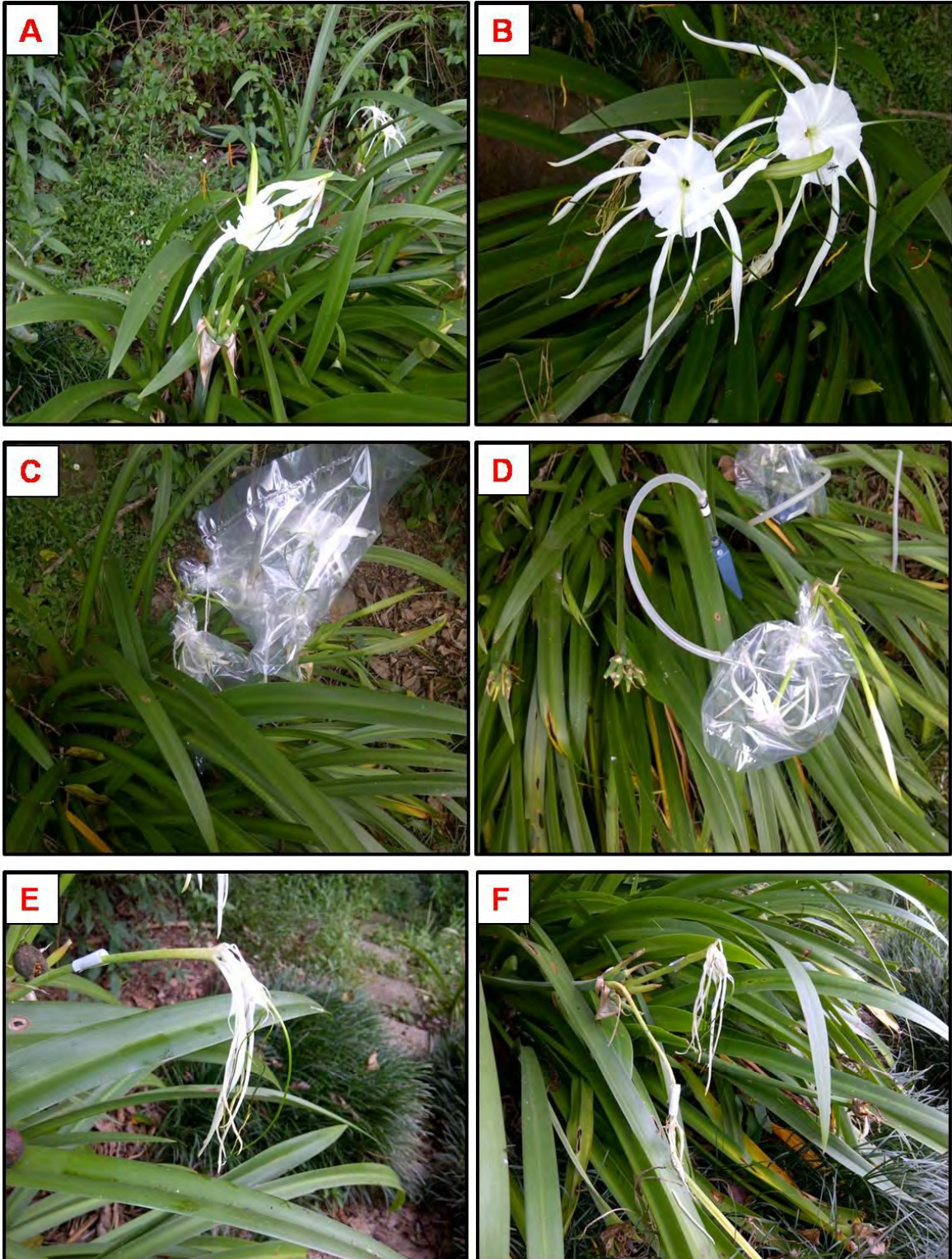


Figure 1. *Hymenocallis littoralis* during (A) the flower opening, (B) the freshly opened stage, (C) flower enclosed in oven bags to accumulate volatiles, (D) scent collection, (E) early wilting stage, and (F) late wilting stage.

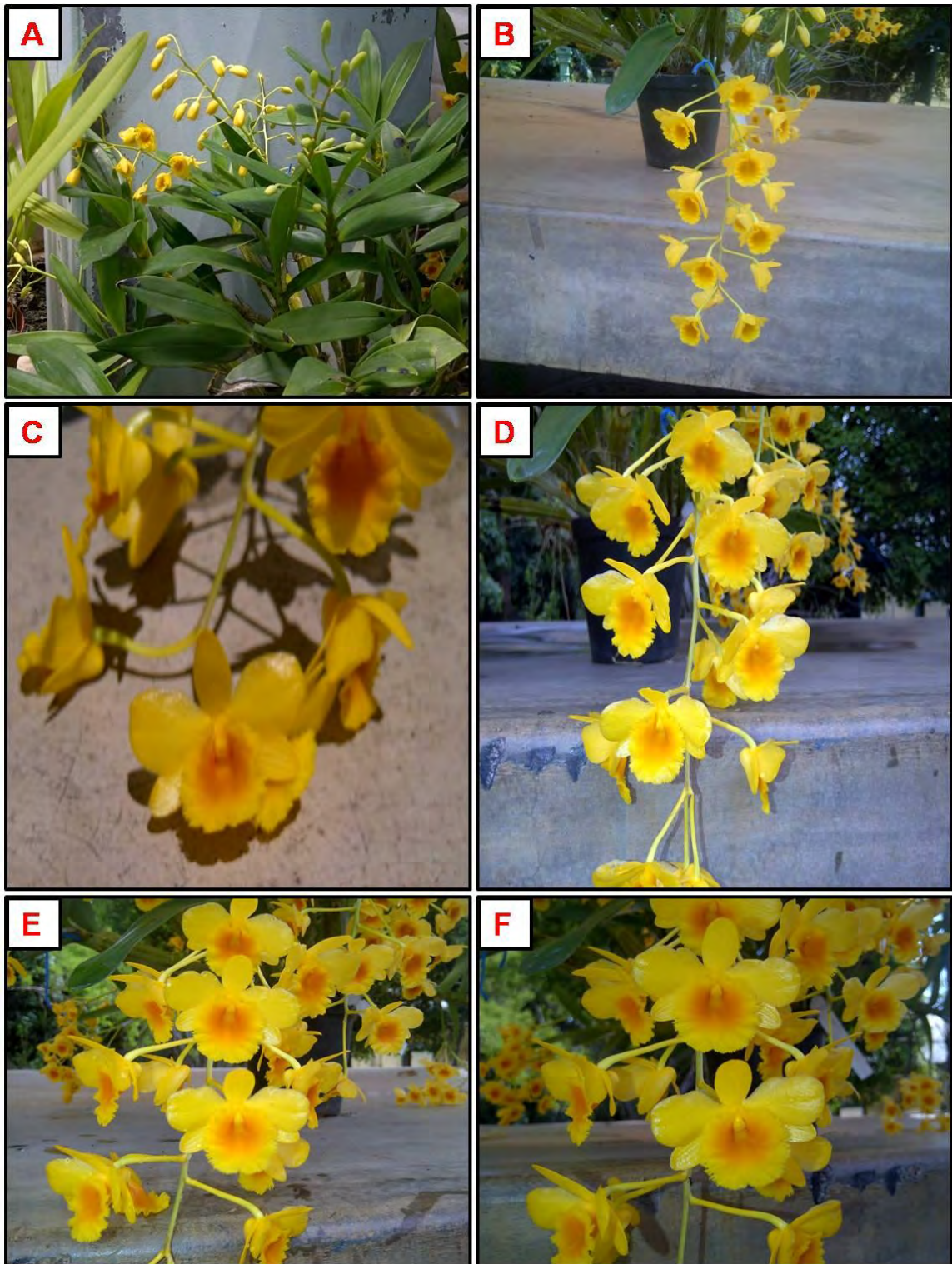


Figure 2. *Dendrobium chrysotoxum* at different flowering stages: (A) bud stage, (B) day 1 of flower opening, (B) day 2, (C) day 3, (D) day 4, (E) day 5, and (F) day 6.

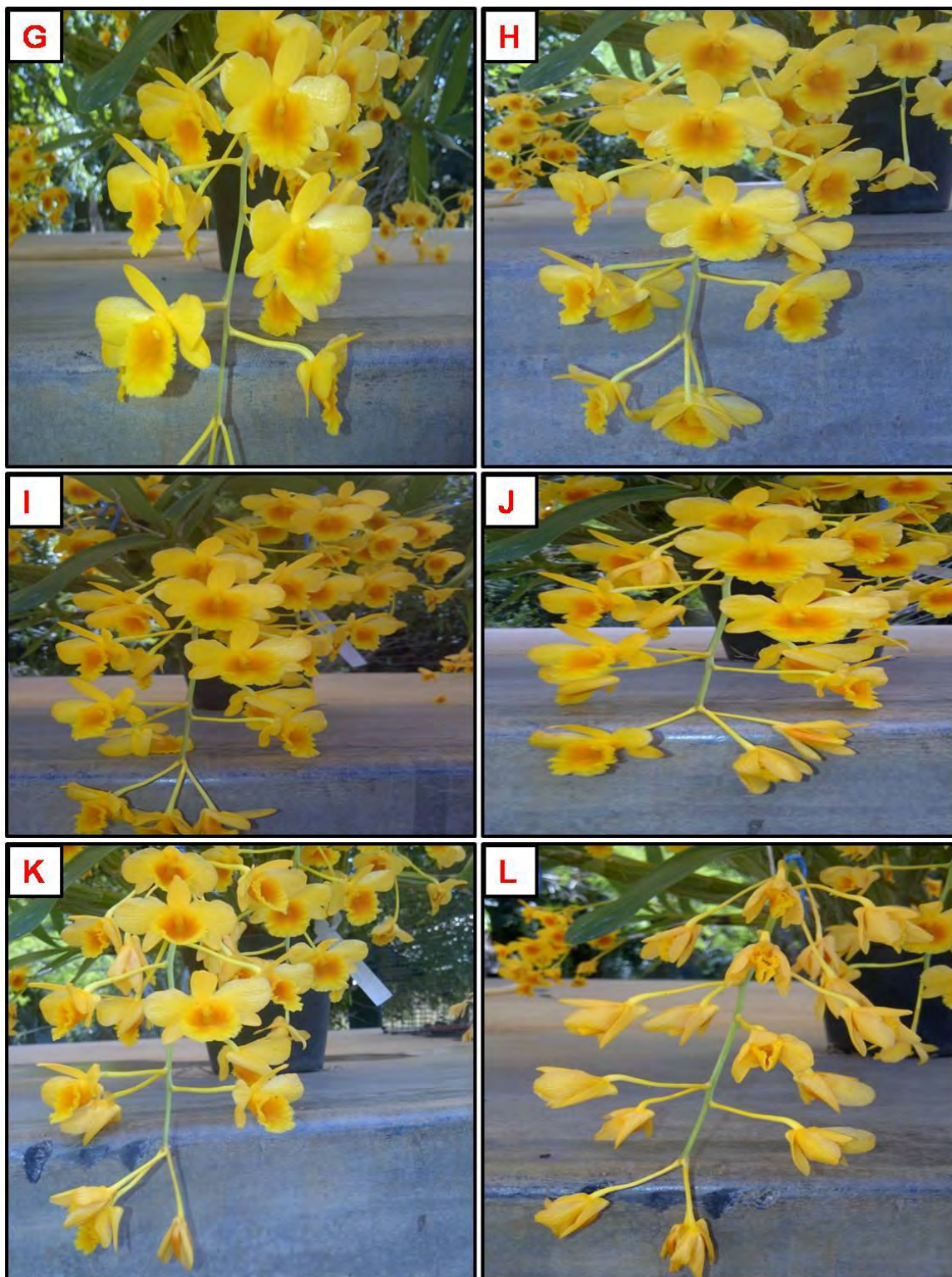


Figure 3. Inflorescences of *Dendrobium chrysotoxum* at different flowering and wilting stages: (G) day 7, (H) day 8, (I) day 9, (J) day 10 and some flowers began to wilt, (K) day 11 more flowers wilted, and (L) day 12 and all the flowers on the inflorescence wilted.



Figure 4. Inflorescences of *Dendrobium chrysotoxum* at different flowering and wilting stages: (M) day 13 and all flowers wilted, (N) a wilted inflorescence of flowers, (O) a single newly opened *D. chrysotoxum* in comparison to (P) which is a completely wilted single flower, (Q) a bagged inflorescence of *D. chrysotoxum* in preparation for scent collection, and (R) collection of scent from the bagged inflorescence using a micro-air sampler and a scent trap.



Figure 5. (A) *Camellia reticulata*, (B) fresh *C. reticulata* flowers collected in the fresh stage from the tree, (C) a single freshly opened *C. reticulata* flower, (D) a bagged *C. reticulata* flower, (E) scent collection from *C. reticulata*, (F) a wilting *C. reticulata* attached to the tree, (G) a completely wilted *C. reticulata* flower, (H) bagged *C. reticulata* flowers, and (I) collection of scent from wilted *C. reticulata*.

Transform: Square root
 Resemblance: S17 Bray Curtis similarity

2D Stress: 0.18

Day

1

2

3

4

Transform: Square root
 Resemblance: S17 Bray Curtis similarity

2D Stress: 0.16

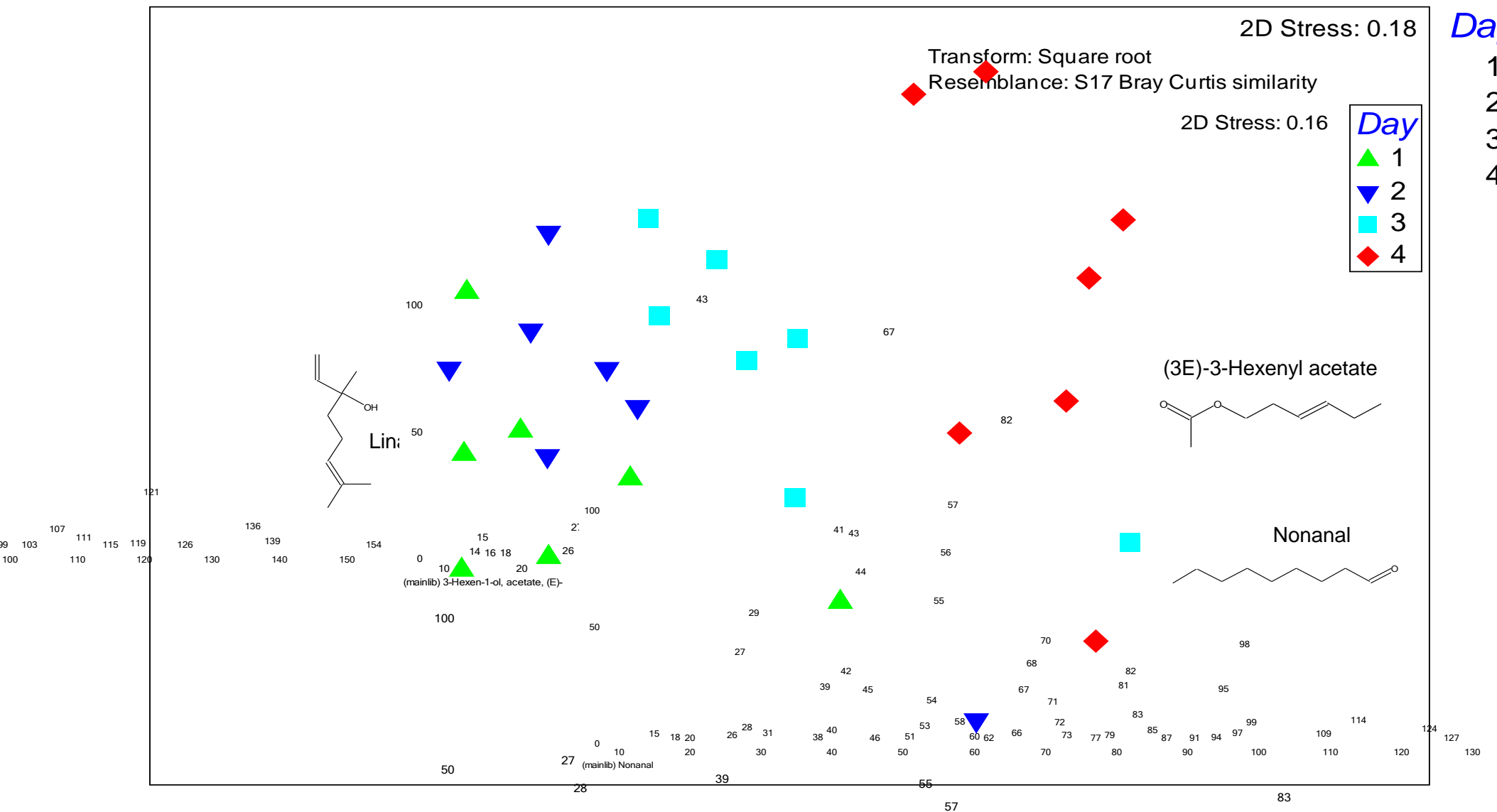
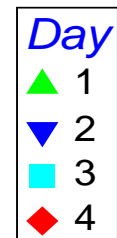
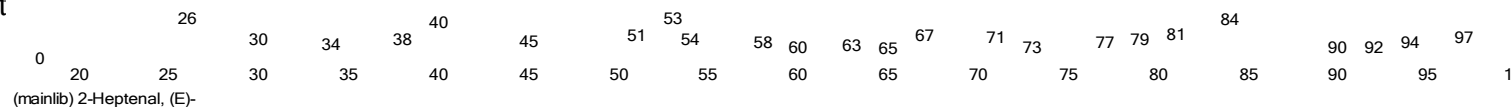
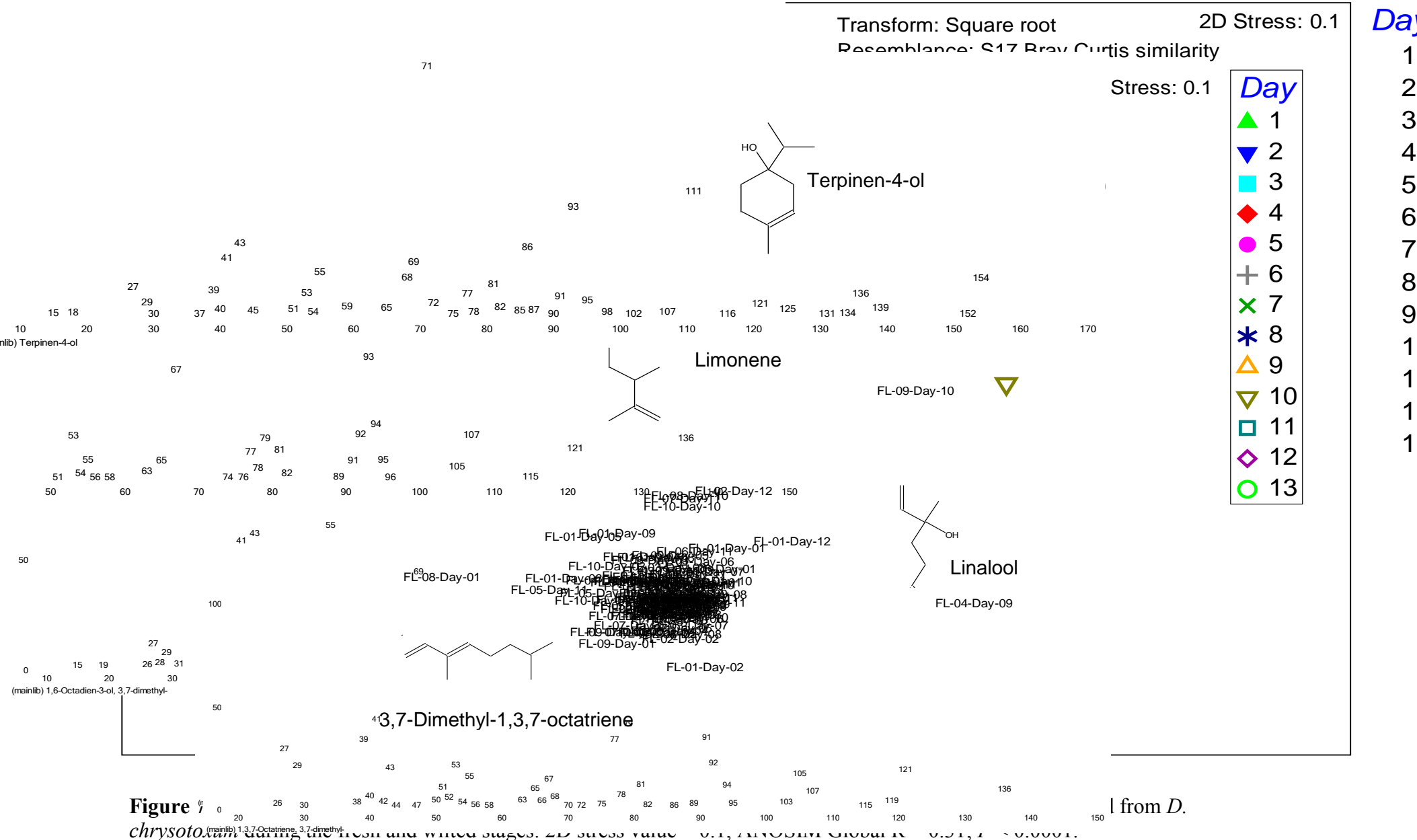


Figure 6. Non-metric multidimensional scaling (NMDS) plot showing the relationship between chemical compounds emitted from *H. littoralis* from t



82

Transform: Square root
 Resemblance: S17 Bray Curtis similarity



Dimension 2

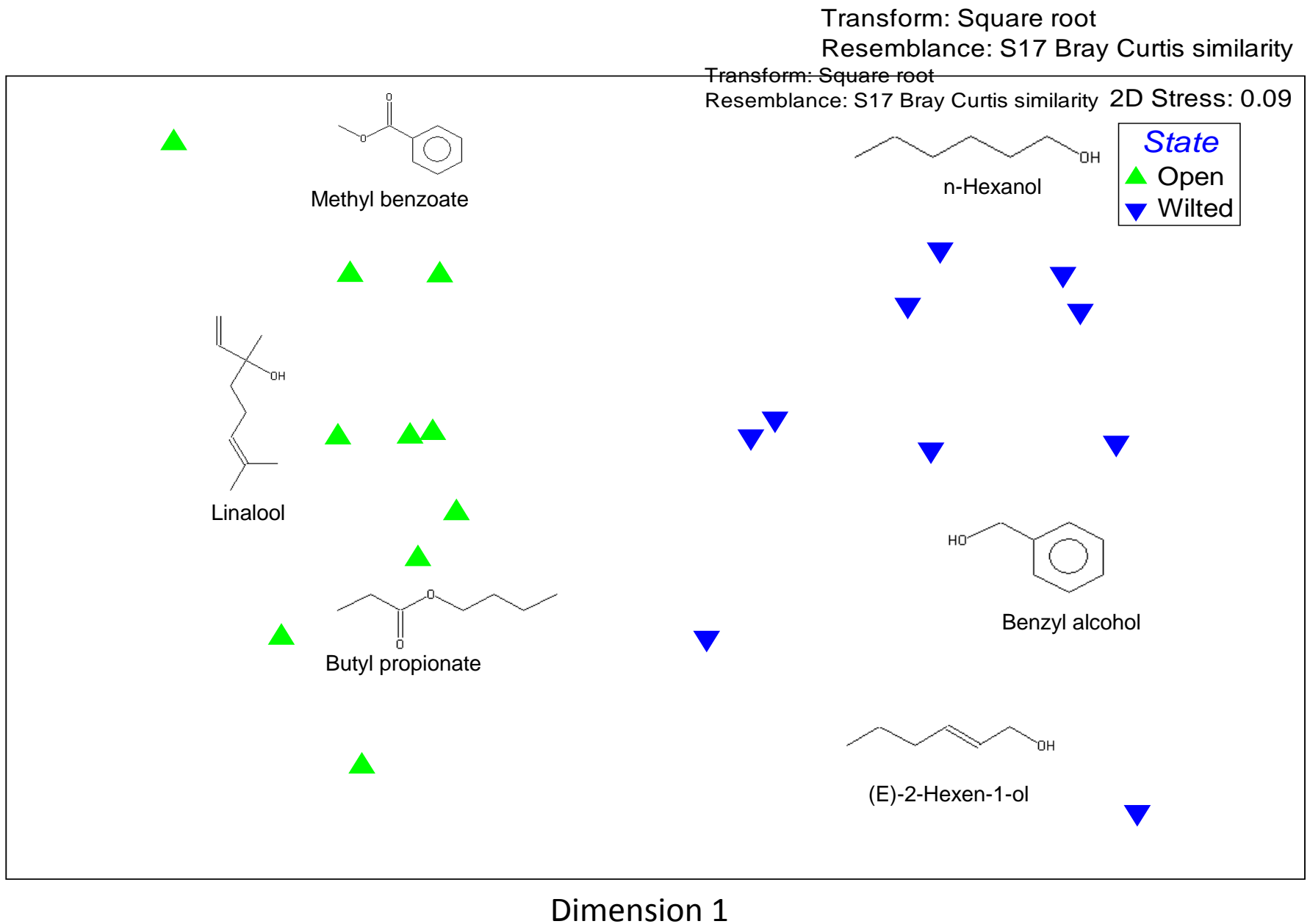


Figure 8. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the variation of compounds emitted from open (fresh) and wilted *C. reticulata* flowers. 2D stress value = 0.09, ANOISM Global R = 0.81, $P < 0.0001$.

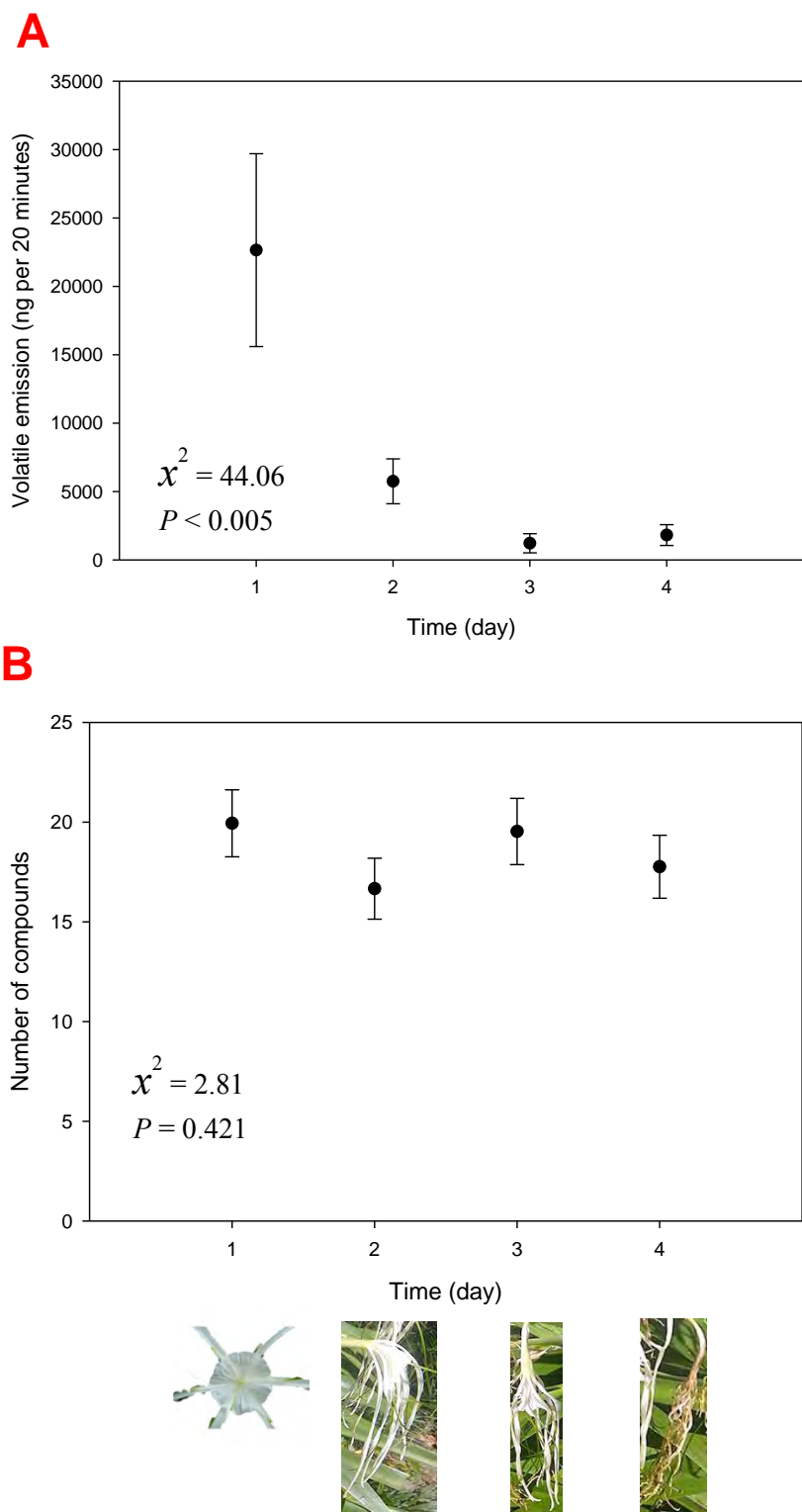


Figure 9. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *H. littoralis* during its life cycle.

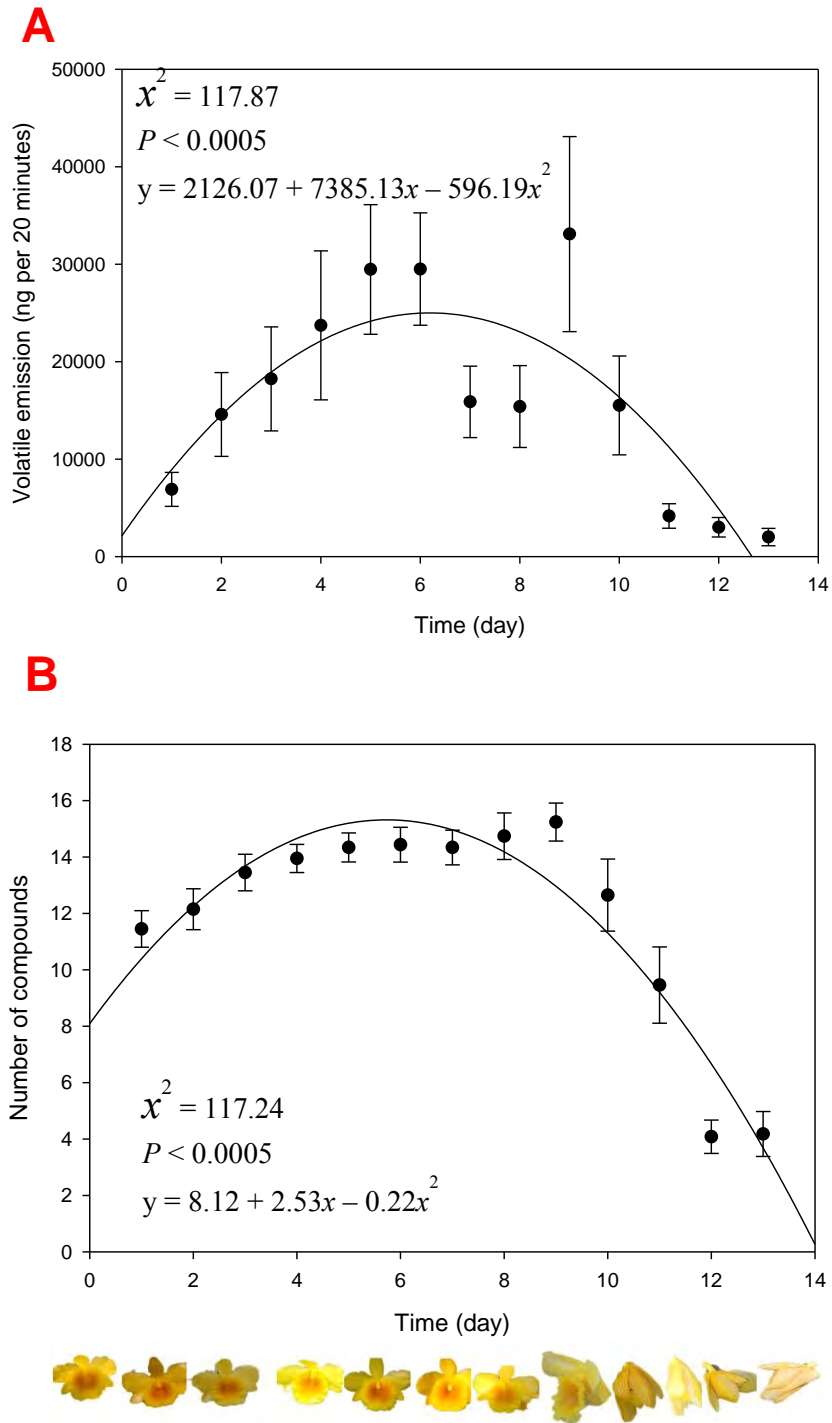


Figure 10. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *D. chrysotoxum* during its life cycle.

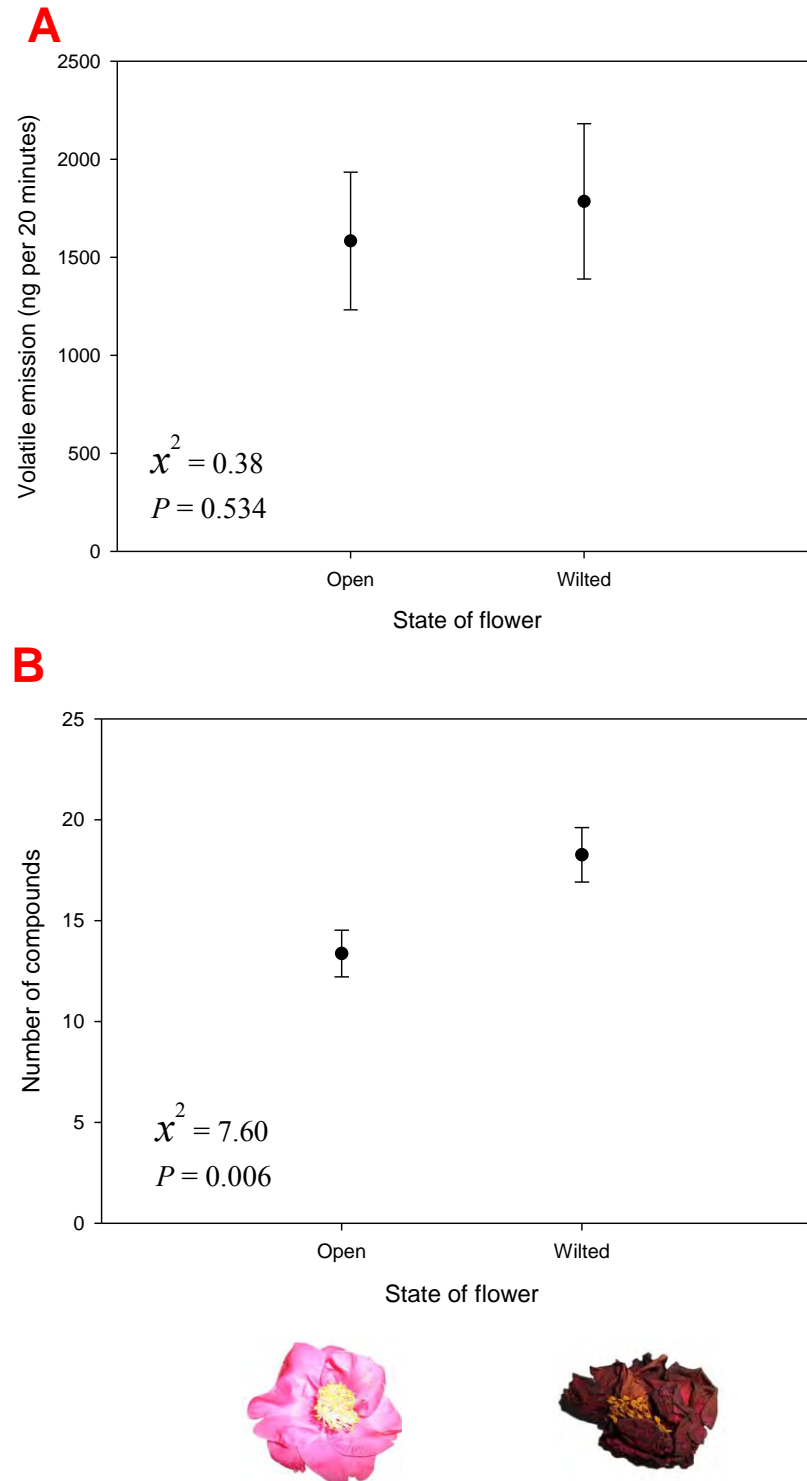


Figure 11. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *C. reticulata* during its life cycle.

Table 1: Relative amounts (%) and the standard deviation of compounds identified by GC-MS from headspace samples of *Hymenocallis littoralis*, *Dendrobium chrysotoxum* and *Camellia reticulata*. Scent compounds are listed according to compound class and Kovats retention index (KRI). Compounds only contributing $\geq 1.0\%$ were included.

Compound	RI Kovats	<i>Hymenocallis</i> <i>littoralis</i>	<i>Dendrobium</i> <i>chrysotoxum</i>	<i>Camellia</i> <i>reticulata</i>
Fatty acid derivatives				
Hydrocarbon alkanes				
3-Ethyl-hexane	752	5.26±11.94	—	—
4-Methyl-octane	862	7.35±21.03	—	—
2,4-Dimethyl-1-heptene		—	8.18±19.99	—
Hydrocarbon aldehydes				
Butanal, 3-methyl-	652	2.89±8.59	—	—
Pentanal	707	1.20±4.63	—	—
Hexanal	807	2.69±6.27	—	—
Heptenal	903	1.21±2.46	—	—
Nonanal	1102	1.46±3.72	—	—
Decanal	1202	1.20±2.21	—	—
(<i>E</i>)-2-Hexenal		—	—	1.68±5.10
Hydrocarbon alcohols				
2-Methyl-3-pentanol		1.05±2.19	—	—
2,3-Butanediol	806	4.75±16.65	—	—
1-Butanol	1119	—	—	1.99±5.32
Unidentified hydrocarbon alcohol	1176	—	1.05±5.49	—
2-Heptanol	1307	—	—	1.18±1.77
n-Hexanol	1316	—	—	2.26±3.38
Hydrocarbon ethers				
Isobutyl ether		—	—	1.41±2.41
Hydrocarbon esters				
(3 <i>E</i>)-3-Hexenyl acetate	990	4.21±9.30	—	—
Butyl acrylate		—	—	6.95±6.56
Octyl acetate	1210	—	3.45±4.30	—
2-Ethyl-1-hexyl acetate	1420	—	—	1.92±3.08

Table 1. Continued.

Compound	RI Kovats	<i>Hymenocallis littoralis</i>	<i>Dendrobim chrysotoxum</i>	<i>Camellia reticulata</i>
Monoterpenes				
α -Pinene	928	—	7.00 \pm 12.88	—
β -Myrcene	992	—	33.40 \pm 20.75	—
Limonene	1018	—	11.53 \pm 10.46	—
2,6-Dimethyl-7-octen-2-ol	1033	1.34 \pm 1.97	—	—
3,7-Dimethyl-1,3,7-octatriene	1049	1.53 \pm 2.10	15.62 \pm 15.61	—
Linalool	1101	9.27 \pm 10.43	8.65 \pm 6.63	13.48 \pm 7.01
Unidentified Monoterpene	1107	6.16 \pm 18.22	—	—
2-Methyl-6-methylene-1,7-octadien-3-one	1117	1.11 \pm 5.88	—	—
(<i>Z</i>)-Verbenol	1143	—	1.66 \pm 7.10	—
Terpinen-4-ol	1179	—	2.29 \pm 7.25	—
(<i>Z</i>)-Geranyl acetate	1364	—	2.14 \pm 2.49	—
(<i>Z</i>)-Linalool oxide (furanoid)	1423	—	—	47.41 \pm 20.67
(<i>E</i>)-Linalool oxide (furanoid)	1425	—	—	2.39 \pm 1.29
Borneol	1675	—	—	3.30 \pm 14.20
(<i>E</i>)-Linalool oxide (pyranoid)	1720	—	—	8.84 \pm 4.70
Irregular Terpene				
6-Methy-5-hepten-2-one	986	3.33 \pm 6.76	—	—
Benzenoid containing compounds				
Benzyl alcohol	1030	4.41 \pm 7.98	—	—
Nitrogen containing compound				
(1 <i>Z</i>)-2-Methylbutanal oxime	810	11.58 \pm 16.92	—	—
Unknowns				
Unidentified compound	1104	5.81 \pm 12.36	—	—
Unidentified compound	668	1.29 \pm 5.86	—	—
Miscellaneous				
1,1-Dimethyl-3-methylene-2-vinylcyclohexane	1071	5.25 \pm 13.21	—	—
(3 <i>E</i> ,5 <i>E</i>)-2,6-Dimethyl-1,3,5,7-octatetraene	1130	—	1.41 \pm 2.14	—

Chapter 4

Volatile organic compounds emitted during the ripe and overripe fruit stages of *Musa acuminata* (banana), *Pyrus communis* (pear), and *Rothmannia globosa*

Abstract

Fruits emit a wide range of volatile organic compounds (VOCs). The production of VOCs in many fruits is linked to their ecological role as chemical cues to attract seed dispersers. In addition, VOCs may also serve as a chemical defence against herbivores, and pathogenic microorganisms. Fruit development is characterized by different stages (e.g. unripe, ripe and overripe) and these stages are characterized by different VOC emissions often characteristic for each stage. Furthermore, the dispersal of seeds away from the parent plant during its right developmental stage is vital for their productive success of the plant. Temporal changes in the VOC composition between different stages may act as indicators for seed dispersers when fruits are ready for consumption, unripe, or non-palatable/rotten. However, relatively little is known about the compounds that are released during older, rotting fruit stages. The aims of the current study were therefore to investigate the temporal changes in the VOC composition during the ripe and overripe stages of *Musa acuminata* (banana), *Pyrus communis* (pear), and *Rothmannia globosa*. Significant differences in the VOC emission were found. Different compounds were found for each of the species during the overripe stage. Acetoin and 2,3-butanediol were the compounds which contributed to the odour of *M. acuminata* during the overripe stage. In *P. communis* the overripe stage was characterized by acetic acid, 1-butanol, 3-methyl-ethyl acetate, and acetoin. In *R. globosa* butyl acrylate (butyl 2-propenoate), benzaldehyde, 1-butanol were found in the overripe stage. The ecological role of temporal changes in VOCs between different developmental stages of fruits for seed dispersal is discussed.

Keywords: VOCs, ripe fruit, overripe fruit, microorganism, fermentation, chemical signal

4.1. Introduction

Fruits contain high amounts of nutrition and are consumed by vertebrates. Fruits release blends of volatile organic compounds (VOCs) to attract animals which facilitate the dispersal of seeds away from the parent plant, thereby increasing the reproductive success of the plant (Tewksbury, 2002). Fruits can be divided into different ripening stages: unripe, ripe and overripe. These stages experience temporal changes in the emission of VOCs. During the unripe stage, fruits are green and unpalatable, and they are dominated by the emission of green leaf volatiles (GLVs) that are accompanied by other secondary metabolites (Cipollini and Levey, 1997). Short-chained hydrocarbon -aldehydes and -alcohols are responsible for the fresh green smell in unripe fruits, the concentration of these compounds decrease with ripening (Rodriguez et al., 2013). The main function of the emission of VOCs in green unripe fruit is to deter all potential consumers, microorganisms and pre-dispersal seed and pulp predators (Cipollini and Levey, 1997). The consumption of unripe fruits may negatively affect seed dispersal because during the early stages of fruit development seeds are not fully developed. Fruits that are unripe and contain secondary compounds that make them unpalatable and therefore seed dispersers are deterred (Valburg, 1992).

Fruits ripening is correlated with a change in the emission of specific VOCs in order to attract seed dispersers (Rodriguez et al., 2013). In addition to VOC changes, ripening is also accompanied by a change in visual appearance (colour), texture, size. The production of carbohydrate rich fruit pulp is also accompanied by an increase in fruit size, a change in texture, and colour (Rodriguez et al., 2013, Herrera, 1982, Barry and Giovannoni, 2007, Valburg, 1992). The role of VOCs during the ripe stages is primarily to attract visitors that will consume the fruit with rewarding nutritious tissue including the seed; the seeds are then carried away from the parent plant and are later disposed in conditions that favour germination (Herrera, 1982, Corlett, 2011).

After the ripening stage the fruit progresses into an overripe/rotting stage. Fruits rot as a result of the ripening process. While ripening is associated with an increase in the nutritional value of the fruit, it is also associated with a decrease in chemical defenses against microbial infestations by bacteria and yeasts (Valburg, 1992). Fruits then progress into a state of over-ripening also due to microbial infestation (Buchholz and Levey, 1990, Valburg, 1992). Overripe

fruits are associated with an increase in the emission of alcohols and esters (Dominy, 2004). The process of over ripening in fruits is merely the degradation of the sugars of fruits. Sugars are naturally produced in fruits, and they provide the main substrate for fermentation (Dudley, 2000). The pulp of the fruit is often the main source of sugar and it also forms a reward to frugivores which disperse seeds (Dudley, 2000). Sugar in ripe fruits range from 61% of the fruit mass (Dudley, 2000). Fruits are more vulnerable to the interaction of microorganisms once they have ripened since ripening increases the nutritional value but it also reduces the chemical defenses that prevent fungi from attacking the sugars (Dudley, 2000, Valburg, 1992). The growth of microorganisms results in the breaking down of cell membranes (Rogiers et al., 1998).

Fermentation of fruit can take place via aerobic and anaerobic pathways. Fermentation is essentially the consumption of sugars by microorganisms like yeasts and ethanol is usually an end product (Levey, 2004). Aerobic pathways are described by the oxidation of ethanol by yeasts or *Acetobacter* bacteria, resulting in acetic acid (vinegar).

While not many studies have investigated the emissions of VOCs from late stages of fruits it is to be expected that changes in the VOC composition might affect behavioural responses of animals (including seed dispersers). The aim of this study was to determine the temporal changes in the emission of VOCs of three fruit species: *Musa acuminata* (banana), *Pyrus communis* (pear), and *Rothmannia globosa* during the ripe, overripe/rotting stages. These species were selected since they were available, commonly consumed by vertebrates and also due to their varying ripening times.

4.2. Materials and methods

4.2.1. Study species

Commercially purchased *Musa acuminata* Colla (Musaceae) and *Pyrus communis* L. (Rosaceae) from Pick & Pay supermarket were used for the laboratory experiments. In addition, fruits of *Rothmannia globosa* (Hochst) Keay (Rubiaceae) which were naturally occurring in the Botanical Garden at the University of KwaZulu-Natal, Pietermaritzburg, South Africa, were investigated. To investigate the changes in the emission of fruit VOCs during the different ripening stages,

scent samples of the selected species were collected. Ten *M. acuminata* and *P. communis* were placed in the laboratory under room temperature and scent samples were collected twice a week. *M. acuminata* volatiles were collected over a four week period until they were completely rotten (**Figure 1** and **Figure 2**). Scent samples from *P. communis* were collected over an eight week period, every third/fourth day depending on visual changes in the texture and colour (**Figure 3** to **Figure 7**). In *R. globosa* the sampling scheme of fruit VOCs was different. Fruits were found in ripe and overripe stages and 10 samples of each stage were collected and sampled (**Figure 8** to **Figure 9**).

4.2.2. Volatile sampling

VOC samples for all the species were collected using dynamic headspace collection methods for subsequent analysis via gas-chromatography-mass spectrometry (GC-MS). Fruits of *M. acuminata*, *P. communis* and *R. globosa* were bagged using Nalophan® bags (Kalle GmbH, Wiesbaden, Germany). Before sampling, fruits were bagged for 20 minutes to allow for accumulation of volatiles. A scent trap, consisting of a micro vial filled with 1 mg of Carbotrap® and 1 mg of Tenax®, was then inserted into each polyacetate bag. Air from these bags was then pumped through the trap with a micro-air sampler (Supelco PAS-500) at a flow rate of 50 ml/min for 20-30 minutes. Samples were stored in a freezer at -20°C until analysis. The micro vials/scent traps that were used to collect the fruit volatiles were conditioned in the laboratory prior to the collection of the fruit scent by washing them with acetone and methanol and heating them at 220°C in an oven. The collection of VOC samples ended when there was no longer any change in the appearance of *M. acuminata* and *P. communis* fruits. Controls were taken on every sampling event in order to determine ambient VOCs.

In addition, the collection and analyses of VOC samples also result in the production and release of plant derived chemicals and microbial VOCs. For the purpose of this study, all the VOCs which were found in the samples were recorded. During the rotting stages of the fruits, the microbial VOCs that were released from the fruits were more apparent in the samples.

A total of 80 samples were collected of *M. acuminata* and 140 for *P. communis* from the fresh to the rotted stage. Since *R. globosa* was found in the fresh and rotting stage, 20 samples were collected, hence 10 during the fresh stage and 10 during the rotting stage.

4.2.3. Gas chromatography Mass Spectrometry (GC-MS)

GC-MS analysis of fruit samples were carried out using a Varian CP-3800 GC (Varian, Palo Alto, California) with a 30 m x 0.25 mm internal diameter (film thickness 0.25 μm) Alltech Carbowax column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70eV (Shuttleworth and Johnson, 2010). For analyses scent traps were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device (Amirav and Dagan, 1997). Samples of *M. acuminata*, *P. communis* and *R. globosa* were run using a Carbowax column. Compounds were identified using the Varian Workstation software with the NIST 2011 mass spectral library (NIST/EPA/NIH Mass Spectral Library, data version: NIST 2011, MS search software version 2.0 d). Identification of compounds were verified using the retention times of authentic standards and published Kovats indices. The absolute scent emission rates were calculated using the peak surface area of a known amount of methyl benzoate as a reference, hence one nanolitre of methyl benzoate was injected into a thermodesorption cartridge and this was run in the GC-MS, this was repeated three times and an average of the repeats were calculated to determine the average total ion count per nanogram of methyl benzoate. Compounds present at similar abundance in the controls were considered to be contaminants and excluded from the analysis. Other settings of the GC-MS were according to Shuttleworth and Johnson (2010).

4.2.4. Statistical analyses

Primer version 6 (Primer E) was used to determine the difference in compounds emitted per sample on the different days of sampling. Due to the variation in VOCs emitted per individual

sample the differences in VOC composition of each sample was assessed using percentages of individual compounds, initially the absolute amounts were derived from the total area under the peaks and thereafter the absolute amounts were converted to percentages. Percentage data for compounds were square-root transformed before calculating Bray-Curtis similarities to detect similarities among samples. A two-dimensional representation of the data was obtained using non-metric multidimensional scaling (NMDS). To evaluate how well the particular configuration produces the observed distance matrix the stress value was given. According to Clarke and Gorley (2001) smaller stress values indicate a better fit of the produced ordination to the observed distance matrix. In addition to test the significance of differences between compounds produced from the ripe to overripe stages, analysis of similarities (ANOSIM) was used with 10,000 random permutations based on the pair-wise Bray-Curtis similarities between samples (Clarke and Gorley, 2001). The ANOSIM test calculates the test statistic R, assessed by random permutations of the grouping vector to obtain an empirical distribution of R under the null model, as well as a level of significance. To assess the extent to which individual compounds contributed to the dissimilarity of the scent of the fruits from the ripe to the overripe/rotting stages, the SIMPER function in PRIMER was used, with the day of sampling being the factor.

Generalized linear models were used to assess the differences in the emission and number of compounds emitted during the different ripening stages of the fruits. A gamma with log link model was used to assess the differences in the emission of compounds, with time (day) as a subject variable, auto regressive structure was chosen for large data sets (*M. acuminata* and *P. communis*) and exchangeable for a small data set (*R. globosa*) and the means were back transformed. A Poisson with log linear model was used to assess the difference in the number of compounds emitted with time (day) as a subject variable, auto regressive structure was chosen for large data sets (*M. acuminata* and *P. communis*) and exchangeable for a small data set (*R. globosa*) and the means were back transformed.

The curve estimation function in SPSS was used to determine the best fitting regression equations. Least square regression models were fit through the means and the model that displayed the highest R^2 was regarded as the best model.

4.3. Results

4.3.1. Temporal changes in the emission of VOCs from *M. acuminata*

A total of 66 compounds were found in *M. acuminata*. There was a significant difference in the emission (ng) of VOCs during the different sampling days as *M. acuminata* transitioned from a ripe to overripe stage (ANOSIM global $R = 0.586$, $P < 0.0001$, **Figure 10**). Pairwise tests showed that high variability in the emission of compounds occurred from day 16 to 22 of sampling ($R > 0.80$, $P < 0.0001$). Acetoin and 2,3-butanediol only occurred during days 19 and 22 of sampling which were the rotted stages of *M. acuminata* (**Figure 2: I and J**). Changes in colour and texture of *M. acuminata* from the ripe to overripe stage were also observed. During the ripe stage, *M. acuminata* was light yellow in colour, accompanied by a smooth and firm texture (**Figure 1: A to F**). However, as the fruit experienced a transition into the overripe stage it turned black in colour and became very soft (**Figure 2: G to J**). In addition microorganisms were also visible inside of the fruit during the overripe stage (**Figure 2: I to J**).

There was a significant difference in the emission of compounds during the ripening stages of *M. acuminata* ($\chi^2 = 44.123$, $df = 7$, $P = 0.0005$, **Figure 13A**). There was a significant difference in the number of compounds emitted from *M. acuminata* during the transition from the ripe to overripe stages ($R^2 = 0.113$, $\chi^2 = 23.017$, $df = 7$, $P = 0.002$, **Figure 13B**). The best fitting least squares model for the number of compounds emitted from *M. acuminata* was a linear regression (**Figure 13B**).

4.3.2. Temporal changes in the emission of VOCs from *P. communis*

A total of 59 compounds were found in *P. communis*. There was a significant difference in the compounds emitted over the sampling days as *P. communis* transitioned from a ripe to overripe stage, (ANOSIM global $R = 0.522$, $P < 0.0001$, **Figure 11**). Pair wise tests showed a higher variability in the emission of compounds from day 40 to 54 of sampling ($R > 0.95$, $P < 0.0001$). Various compounds contributed to the scent of *P. communis* on the different sampling days. The compounds which contributed to the overall scent of *P. communis* during sampling days 1 to 18 were: butyl acetate, hexyl acetate, n-butyl butanoate, 1-hexanol and n-amyl acetate. Sampling

days 22 to 35 comprised of the same set of compounds as described for sampling days 1 to 18, with the addition of ethyl acetate. During the 40th day of sampling, the chemical composition of *P. communis* changed, with an increase in the amounts of ethyl acetate, acetic acid, 3-methyl-1-butanol, phenylethyl alcohol and benzaldehyde that were emitted. There was a variation in the compounds emitted from *P. communis* during the last two sampling days (49 and 54). The main contributing compounds to the scent of *P. communis* on the (i) 49th day of sampling were: acetic acid (23.66%), 1-butanol, 3-methyl- (16.64%), ethyl acetate (13.215%), and acetoin (8.56%), and (ii) 54th day of sampling were: acetic acid (34.16%), acetoin (9.42%), phenylethyl alcohol (8.20%), and benzaldehyde (6.57%). Changes in the colour and texture of *P. communis* were also observed. During the ripe stages, *P. communis* was dark green in colour and had a smooth and firm texture (**Figure 3**). However as the fruit progressed into an overripe stage, it turned into a shade of pale yellow and began to soften (**Figure 4**). During complete over ripening (rotting), the fruits turned black and were very soft (**Figure 5 to Figure 7**).

There was a significant difference in the emission (ng) of compounds during the ripening stages of *P. communis* ($\chi^2 = 203.710$; $df = 13$, $P = 0.0005$, **Figure 14A**). There was a significant difference in the number of compounds emitted from *P. communis* during the transition from the ripe to overripe stages ($R^2 = 0.219$, $\chi^2 = 33.865$, $df = 13$, $P = 0.001$, **Figure 14B**). The best fitting least squares model for the number of compounds emitted from *P. communis* was a linear regression (**Figure 14B**).

4.3.3. Temporal changes in the emission of VOCs from *R. globosa*

A total of 28 compounds were found in *R. globosa*. There was a significant difference in the compounds emitted during the ripe and overripe stages of *R. globosa* (ANOSIM global $R = 0.871$, $P < 0.0001$, **Figure 12**). The compound 1-hexanol contributed 36.13% to the overall scent of *R. globosa* during the ripe stage. The compounds which contributed to the scent of *R. globosa* during the overripe stage included: n-butyl acrylate (26.40%), benzaldehyde (25.75%), 1-butanol (13.47%) and 2-ethyl-1-hexyl acetate (10.49%). *R. globosa* during the ripe stage was brown and firm (**Figure 8C**), however during the overripe stages the fruits turned black and brittle and could be cracked open by pressing it (**Figure 9H**).

There was a significant difference in the emission (ng) of compounds during the ripening stages of *R. globosa* ($\chi^2 = 12.21$, $df = 2$, $P = 0.0005$, **Figure 15A**). There was a significant difference in the number of compounds emitted from *R. globosa* during the transition from the ripe to overripe stages ($\chi^2 = 12.408$, $df = 2$, $P = 0.0005$, **Figure 15B**).

4.4. Discussion

4.4.1. Temporal variation in fruit VOCs

Biochemical, physiological and structural changes in ripening fleshy fruits are due to ethylene production (Barry and Giovannoni, 2007, Lelièvre et al., 1997). Ethylene regulates genes that are involved in the production of enzymes that constitute to fruit ripening (Lelièvre et al., 1997). Fruits can be divided into two major groups, climacteric and non-climacteric fruits, based on respiration and ethylene production during ripening (Lelièvre et al., 1997). In climacteric fruits, a sharp increase in ethylene during the ripening process is responsible for the changes in colour, aroma, flavour, and other biochemical and physiological characteristics (Lelièvre et al., 1997). However, in non-climacteric fruits, the ripening process is ethylene independent (Lelièvre et al., 1997). Temperature and carbon dioxide levels also affect ethylene production in fruits, low temperatures and low levels of CO₂ are said to delay the onset of ethylene production thereby increasing the freshness period of fruit (Lelièvre et al., 1997). *M. acuminata* and *P. communis* which were used for this study both reflect the characteristics of climacteric fruits. It can therefore be assumed that both, *M. acuminata* and *P. communis* experienced a sharp increase in ethylene production which resulted in colour and odour changes (see **Figure 1** to **Figure 8**). *R. globosa* is a non-climacteric fruit and remained fresh for a longer period of time (see **Figure 9**).

According to Levey (2004), smaller fruits which have a high surface to volume ratios will be unlikely to produce large colonies of yeast. However, larger fruits such as *M. acuminata* and *P. communis* which have large amounts of pulp attained from aerobic conditions can produce large amounts of yeasts. In addition, ethanol content differs in lipid rich and sugar rich fruits, *Candida* and *Saccharomyces* are yeasts respectively found in the above stated (Levey 2004 and references therein). The amount of ethanol is higher in sugar rich fruits. The total amount of

fungi which contributes to rotting of fruit is unknown, however 102 taxa on 18 species were reported on wild fruit in Hong Kong (Tang et al., 2003).

This study found that there were differences in the emission of VOCs as *M. acuminata*, *P. communis* and *R. globosa* transitioned from the ripe to overripe stage. There was a gradual transition in the emission of VOCs from *M. acuminata* (**Figure 10**). Green leaf volatiles (GLVs) which occurred in the overripe stage of *M. acuminata* included: 1-hexanol and 3-hexen-1-ol, hence these compounds were plant derived (Ruther, 2000). However, these GLVs contributed very little to the overall odour of *M. acuminata* during the overripe stage. Banana generally gets its characteristic aroma from volatile esters such as isoamyl acetate and isobutyl acetate (**Table 1**), (El Hadi et al., 2013). Fermentation compounds found during the overripe stage of *M. acuminata* included: 1-butanol, 3-methyl-, acetate, isopentyl 2-methylpropanoate, 1-butanol, 3-methyl- and isopentyl 3-methylbutanoate, It could be inferred that these compounds are microbial derived since they are also commonly found in wine, rice, and vinegar and these are often made as a result of the process of fermentation (Cha et al., 2012).

An abrupt change in the emission of VOCs from *P. communis* was observed from day 40 until day 54 of sampling (**Figure 11**). GLVs which were found during the overripe stage of *P. communis* included: 1-hexanol and hexyl acetate. VOCs identified during the ripe stages such as: butyl acetate, hexyl acetate and butyl butanoate (**Table 1**), contributed to the overall pear aroma (El Hadi et al., 2013). VOCs which were found in the overripe stages of *P. communis* included: ethyl acetate, acetic acid and 1-butanol, 3-methyl-, these VOCs are fermentation compounds since they have also been found in wine and vinegar and these products are created by the process of fermentation (Cha et al., 2012).

A clear difference in the emission of VOCs during the ripe and overripe stages of *R. globosa* was seen (**Figure 12**). A number of GLVs were found in samples collected during the ripe stage of *R. globosa*, namely: 1-hexanol, (*E*)-2-hexenal, (*2E*)-2-hexen-1-ol and (*3Z*)-3-hexen-1-ol. The overripe stage of *R. globosa* included: benzaldehyde, 1-butanol, n-butyl acrylate, 2-ethyl-1-hexyl acetate and 5-hepten-2-one, 6-methyl-.

The VOCs that were found in *M. acuminata* and *P. communis* primarily belong to the hydrocarbon esters and hydrocarbon alcohols (**Table 1**). However, the VOCs identified during

the ripe and overripe stages of *R. globosa* belong to the hydrocarbon alcohols and benzenoids (Table 1).

4.4.2. Why is the ripening stage of fruits accompanied by a change in VOCs?

The process of fruit ripening is vital in ensuring reproductive success of plants. The changes in the emission of VOCs during the overripe stage of fruits probably serve as a stop signal to antagonistic animals that could negatively affect the reproductive success. Therefore, fruit ripening and fruit fermentation are discussed with respect to their effects on seed dispersers such as vertebrates.

It is assumed that the rotting of fruit which is initiated by microorganism is not a mutualistic relationship but it is rather predatory (Levey, 2004). Microorganisms consume fruits and make them look unattractive to their vertebrate frugivores which are often utilized by plants as mutualistic partners for the dispersal of seeds (Dominy, 2004, Levey, 2004). Plants which produce fruits are faced with the problem to defend fruits against microbes and at the same time not to repel beneficial seed dispersers by the emission of VOCs that act as a chemical defense against microbes (Levey, 2004). The “directed deterrence” hypothesis states that plants should produce compounds in the fruit that negatively affect the growth of microorganisms and have minimal effects on seed dispersers (Buchholz and Levey, 1990, Levey, 2004, Tewksbury et al., 2008).

According to Janzen (1977) vertebrates and microorganisms compete for the carbohydrate rich fruit pulp. The microorganisms which take over fruits, decrease the nutritional value of fruits and thus vertebrates are deterred from such fruits (Buchholz and Levey, 1990). Janzen’s (1977) original idea that microbes would also benefit from deterring vertebrates by rendering fruits objectionable to larger animals, for example by producing microbial VOCs, seems questionable however. Although it is possible that some of the chemicals produced by microorganisms may have a deterrent effect on larger animals, however, it seems unlikely that this effect has evolved as a result of competition with animals (Sherratt et al., 2006). Using mathematical models Sherratt et al. (2006) showed that the high dispersal rates of microbes and cost for the microbe to produce the fruit spoiling chemicals makes it very unlikely for a spoiling

strategy to persist. Fruit flies also use the VOCs emitted by microbes to locate fruit and as a result, the flies disperse spores onto other foods (Venu et al., 2014).

A study carried out Sánchez et al. (2004) on Egyptian fruit bats (*Rosettus aegyptiacus*) indicated that ethanol concentrations higher than those found in ripe fruit were completely avoided by the bats and did not attract them neither did it act as an appetite stimulant. In addition, the ethanol would provide some source of energy but it does not relate to the caloric value of ingested carbohydrates (Sánchez et al., 2004). Ethanol at high concentrations are used as an odour cue to bats to avoid fruits because at very late stages the fruits are unpalatable (Sánchez et al., 2006). In another study carried out using cedar waxwings, they were offered two types of fruit an: (i) infected and (ii) uninfected fruit of five species, which was randomly placed in the perch (Buchholz and Levey, 1990). The fruits were placed so that at least one spot indicating rotting was visible to the bird. It was found that cedar waxwings showed a preference for uninfected fruits. In more than 50% of the trials, the uninfected fruits were taken first, in 11% of the trials the infected fruits were completely untouched (Buchholz and Levey, 1990). If the birds did consume infected fruits, they would throw up the infected fruits (Buchholz and Levey, 1990).

Levey (2004) concluded that frugivores prefer ripe palatable fruit in comparison to overripe microorganism infested fruits. In primates, the production of ethanol in fruits along with the soft texture, provided information to primates on fruit quality (Dominy, 2004). However, overripe fruits rich in ethanol would probably be consumed by vertebrates if there are no alternative food sources (Dominy, 2004, Sánchez et al., 2008). However, the response to ethanol is very different in some insects. For instance, *Drosophila* spp. fly upwind tracking ethanol plumes in order to locate favorable oviposition sites (Dudley, 2000). Furthermore, in *Drosophila* ethanol may also serve as a appetite stimulant thereby resulting in the consumption of the resources (Sánchez et al., 2004; Dudley, 2000).

4.4.3. Synthesis and future perspectives

Temporal changes in the emission of VOCs of *M. acuminata*, and *R. globosa* during the ripening process were found. Further studies are required to determine the effect that the emission of ripe and overripe VOCs have on seed dispersers. The next step could be to carry out behavioural

experiments with fruit dispersers to test their response to different mixtures of fruit and fermentation volatiles.

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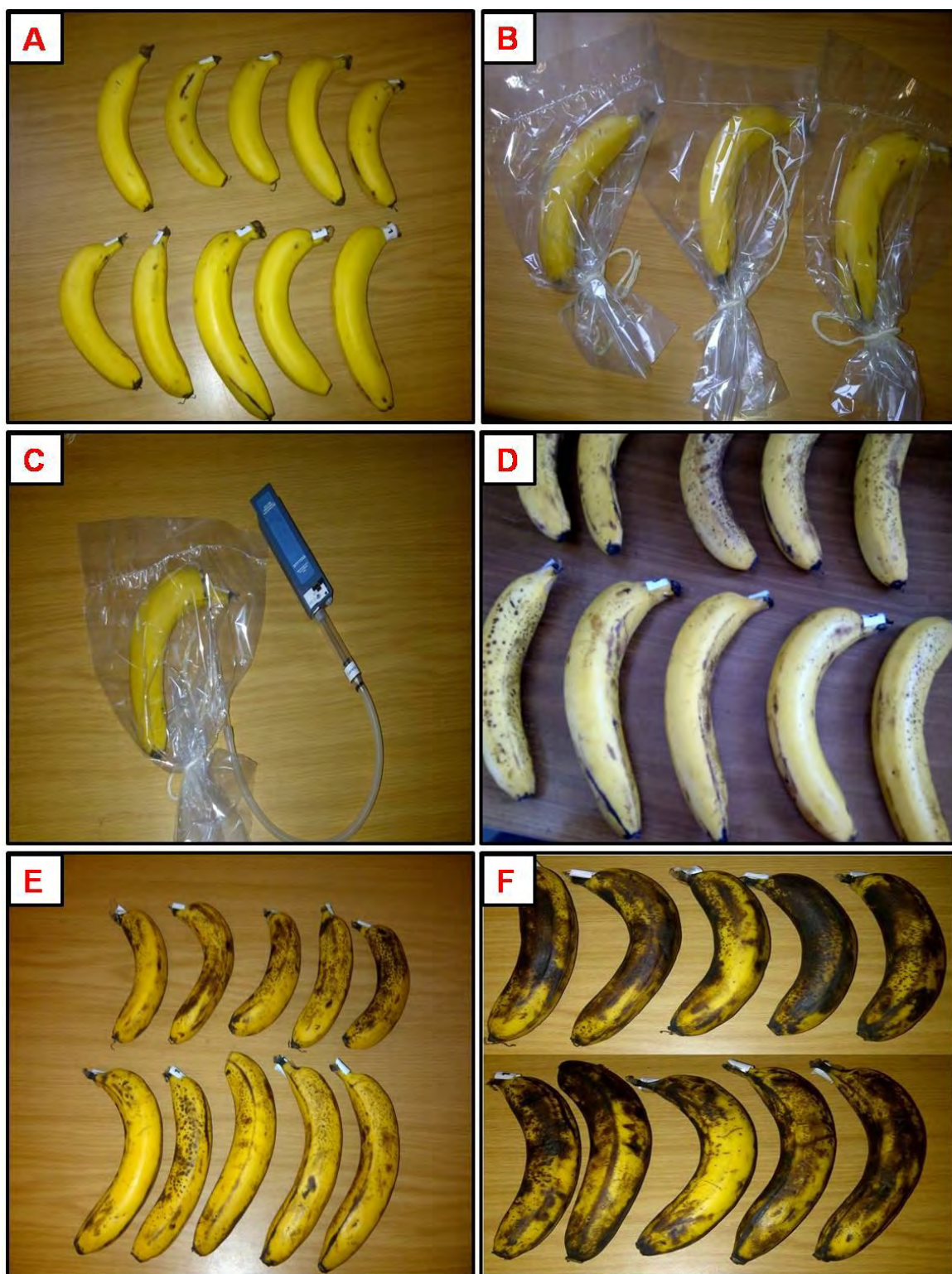


Figure 1. Fruits of *Musa acuminata* on: (A) First day of sampling, (B) *M. acuminata* bagged using polyacetate oven bags to accumulate scent, (C) a micro-air sampler with a scent trap, inserted into the oven bag, *M. acuminata* on (D) day 4, (E) day 7, and (F) day 10 of sampling.

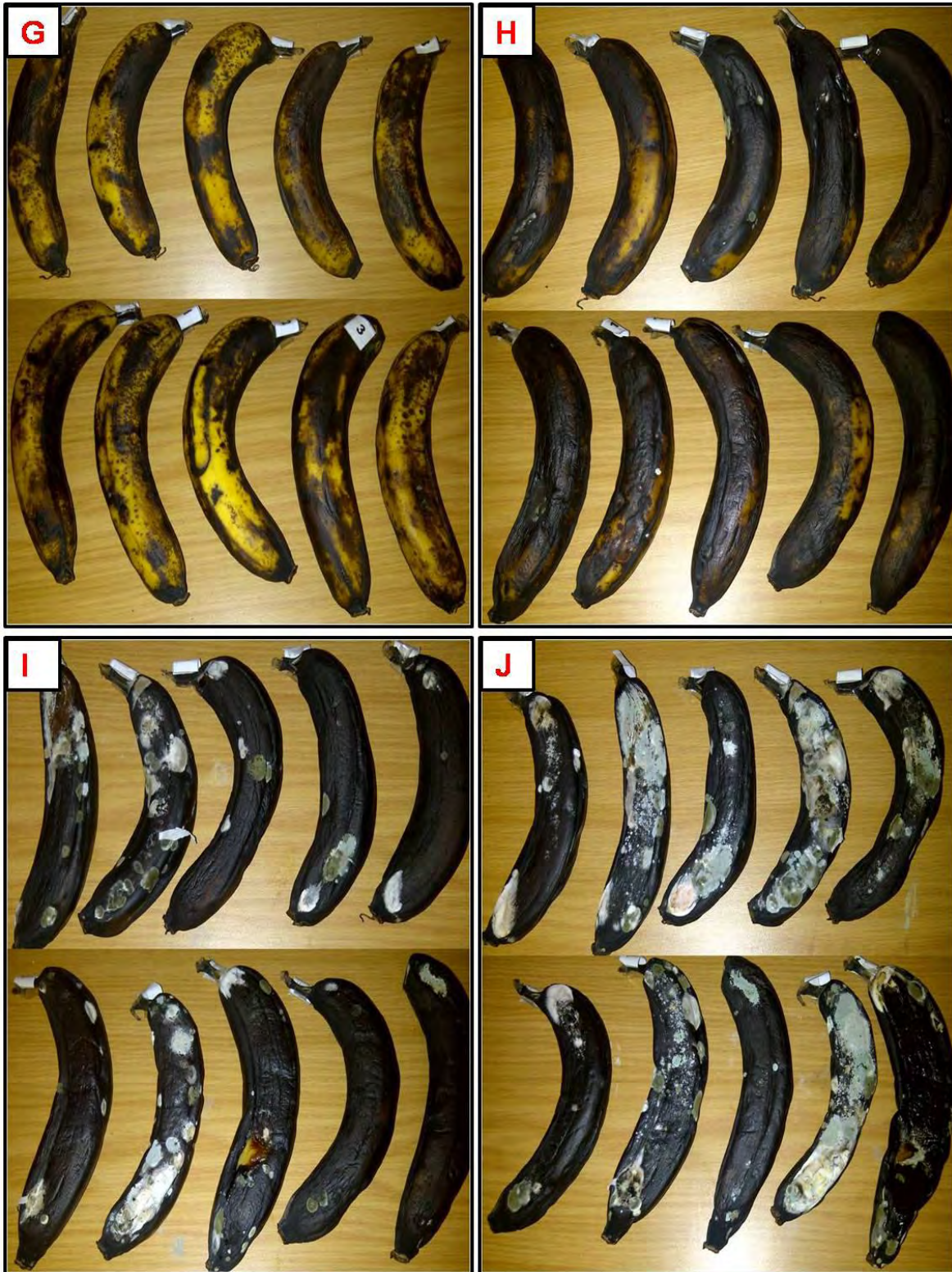


Figure 2. Fruits of *Musa acuminata* on: (G) day 13, (H) day 16, (I) day 19, and (J) day 22 of sampling.

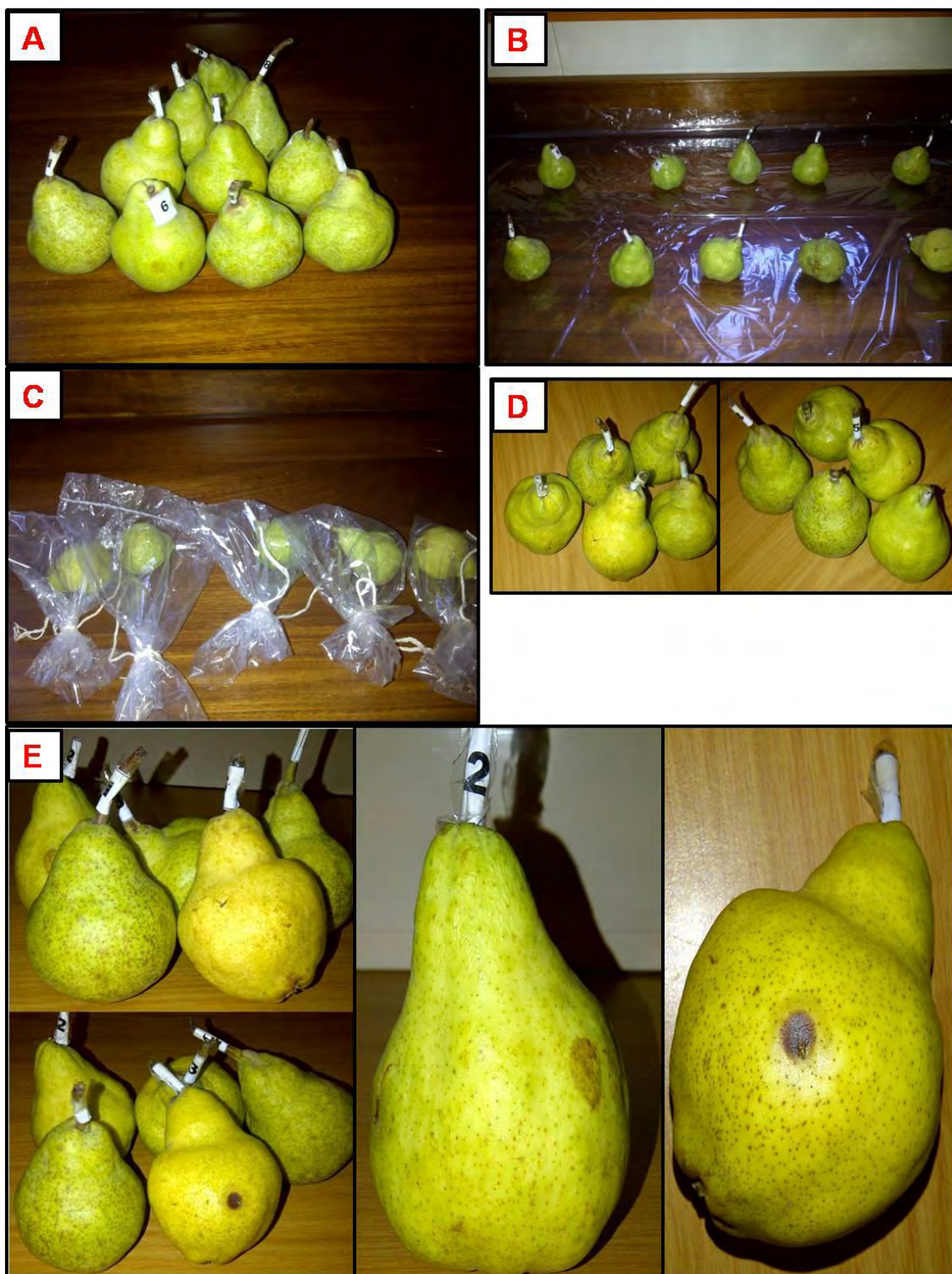


Figure 3. (A) Fruits of *Pyrus communis* purchased in the ripe stage, (B) day 1 of sampling, (C) *P. communis* bagged to collect scent. *P. communis* on (D) day 4, and (E) day 7 of sampling and there were visible signs of microbial infestation on pears number five and two.

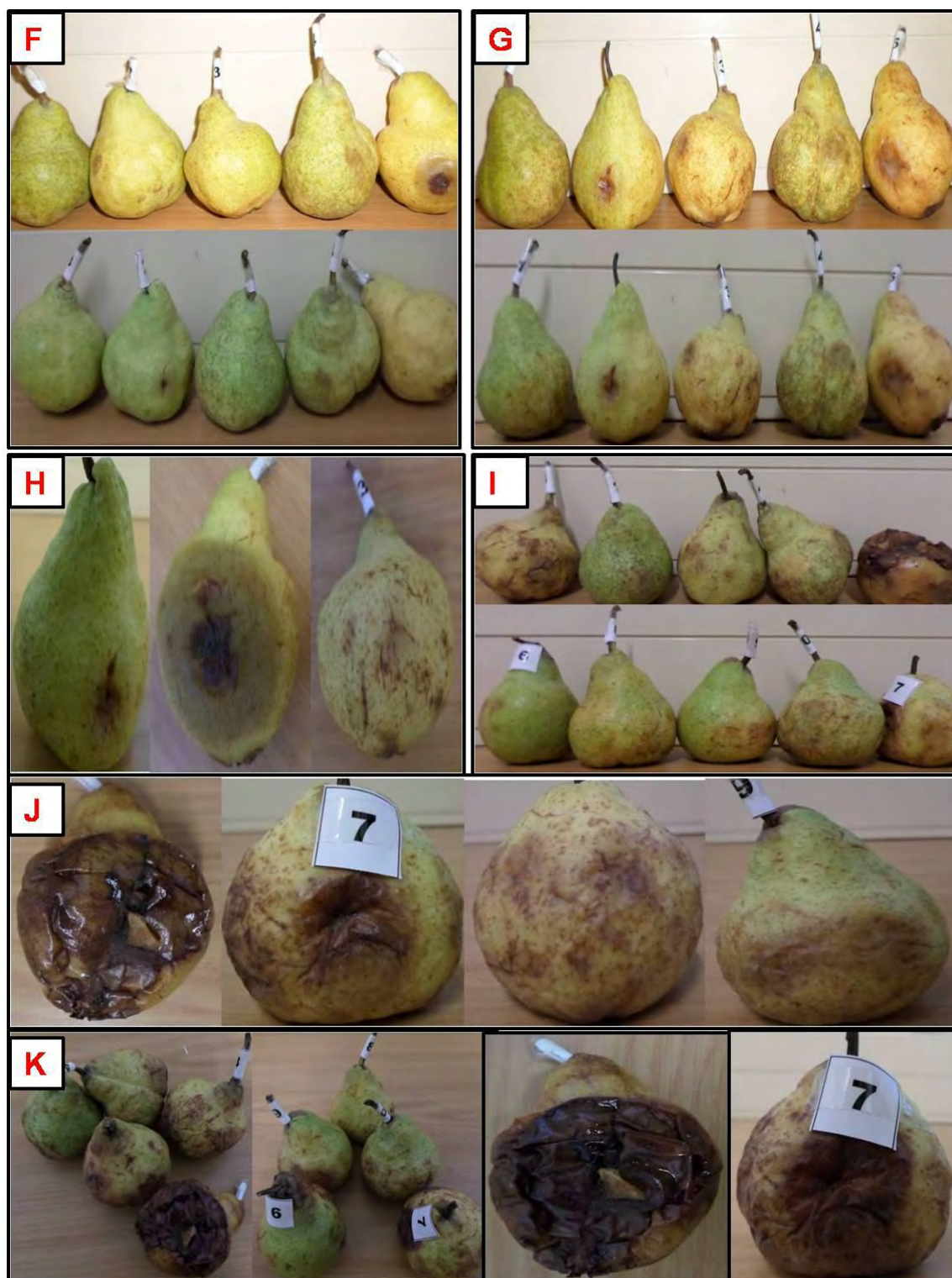


Figure 4. *Pyrus communis* on (F) day 10, (G) day 15, (H) increase in microbial action, (I) *P. communis* on day 18. (J) *P. communis* number five with clear signs of over ripening and microbial infestation. (K) *P. communis* on day 22 of sampling.



Figure 5. *Pyrus communis* on (L) day 25 of sampling accompanied by an increase in ripening, and (M) day 28 of sampling.



Figure 6. Appearance of *Pyrus communis* on (N) day 31, and (O) day 35 of sampling.



Figure 7. *Pyrus communis* on (P) day 40, (Q) day 49 and day 54 of sampling, this marked the end of sampling since there were no change in the condition of the *P. communis* thereafter.

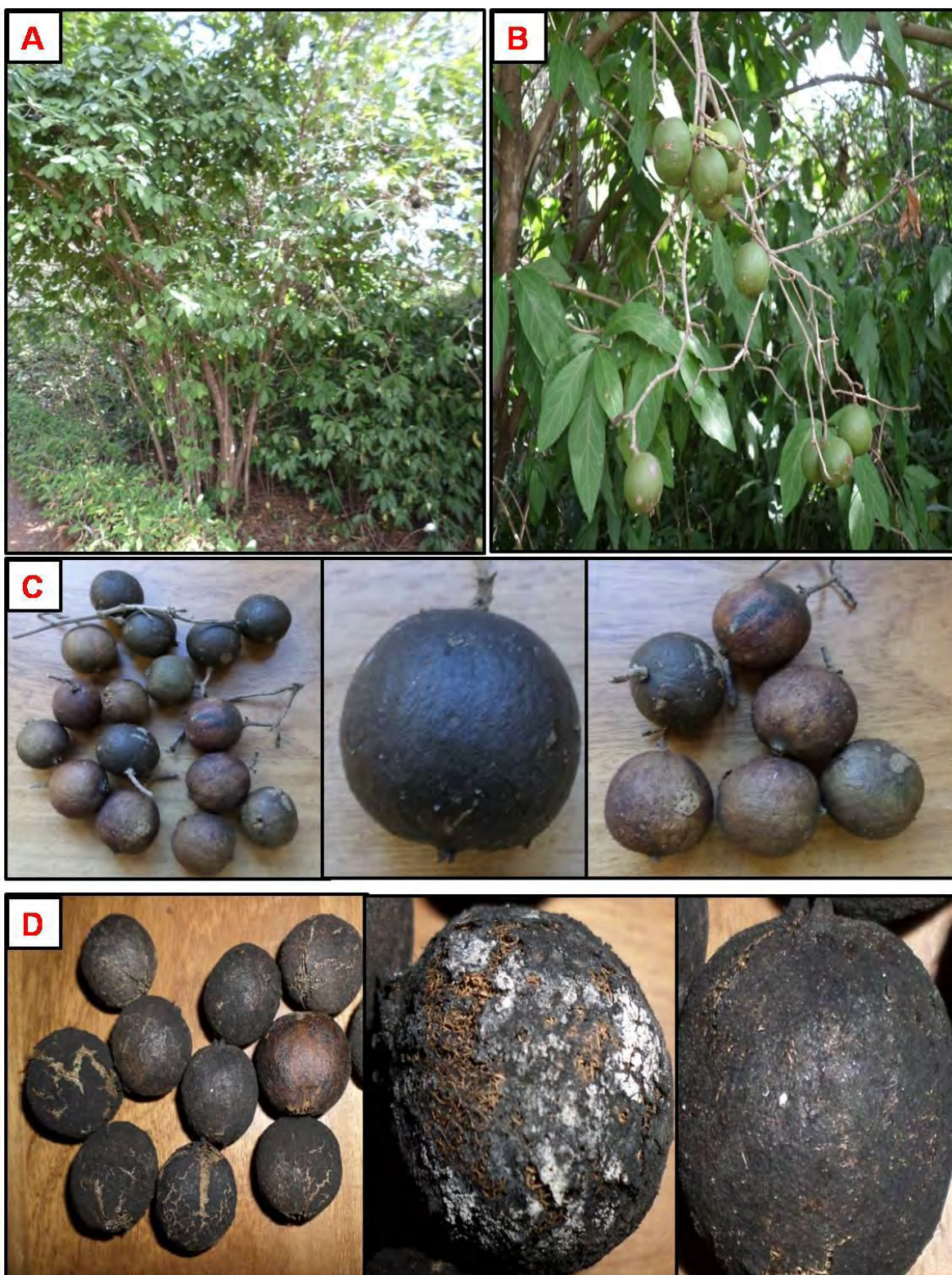


Figure 8. (A) *Rothmannia globosa* tree, (B) unripe fruit on the tree, (C) ripe fruit, and (D) overripe fruit.



Figure 9. (E) Fruits of *Rothmannia globosa* bagged to accumulate scent, (F) collection of scent using a micro-air sampler and a scent trap, (G) internal view of *R. globosa* during the ripe (brown) stage, and (H) internal view of an overripe/rotted *R. globosa*.

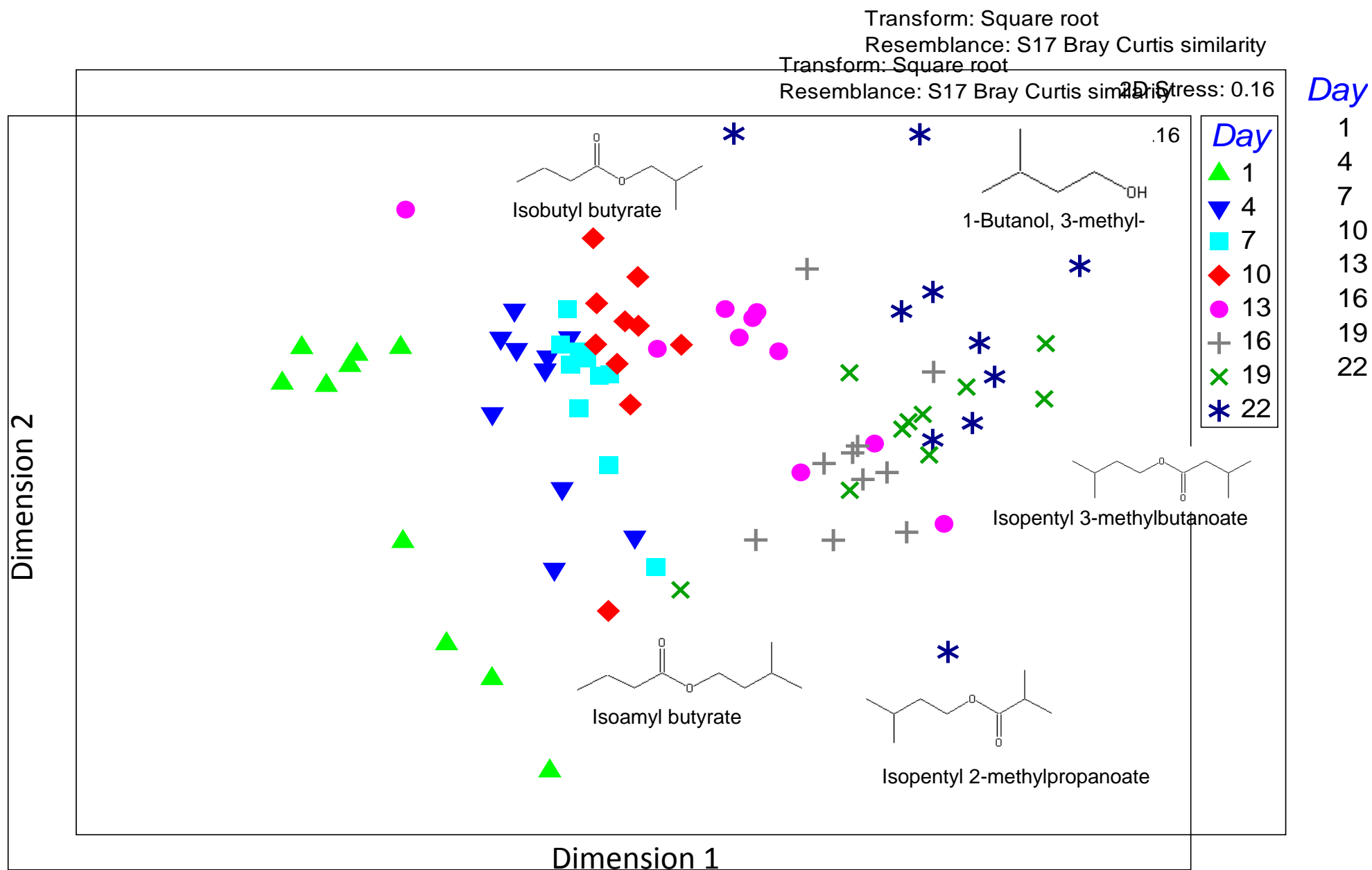


Figure 10. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the variation of compounds emitted from the ripe stage to overripe stage of *M. acuminata*. 2D stress value = 0.16, ANOSIM Global R = 0.586, $P < 0.0001$.

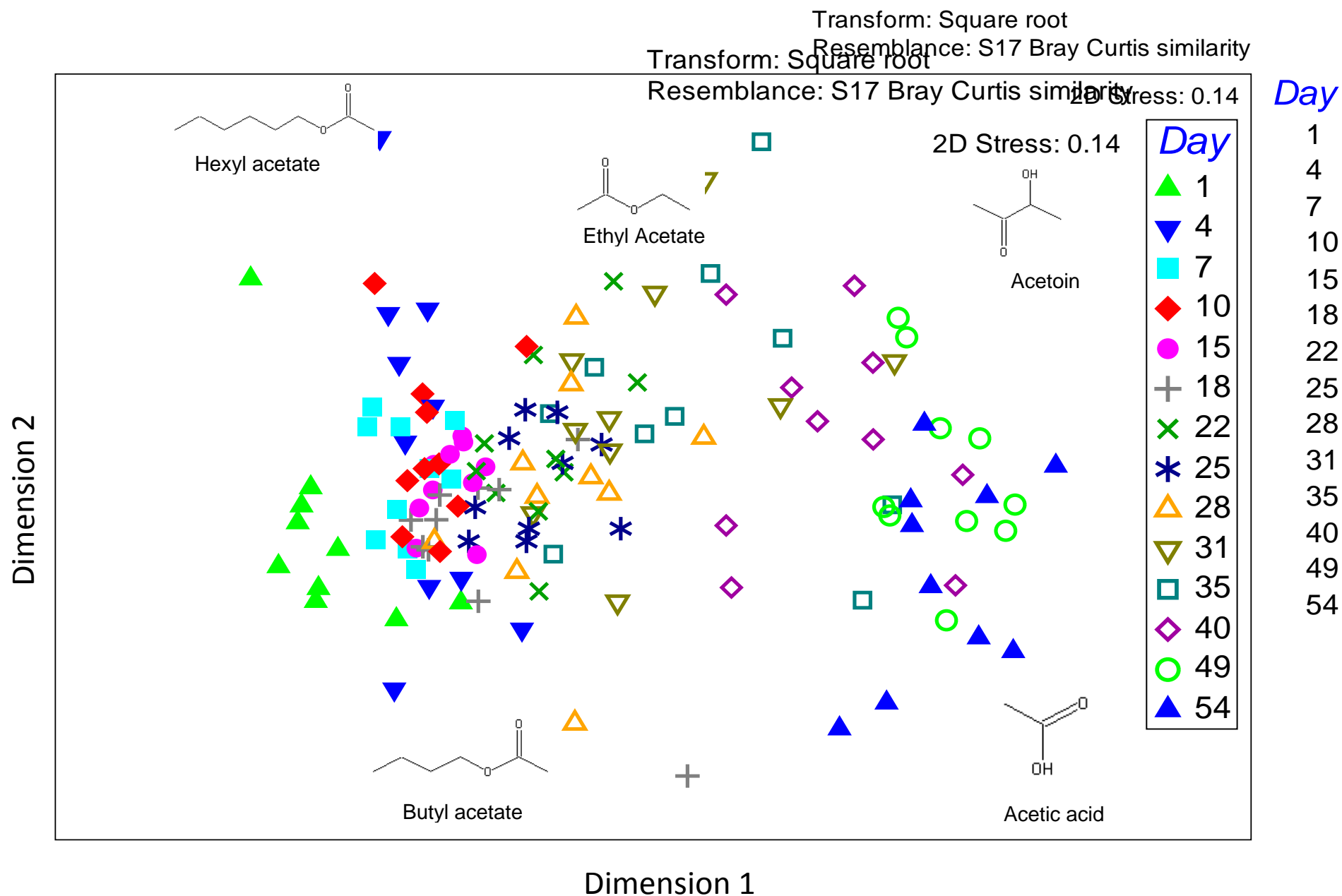


Figure 11. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the variation of compounds emitted from the ripe stage to overripe stage of *P. communis*. 2D stress value = 0.14, ANOSIM Global R = 0.522, $P < 0.0001$.

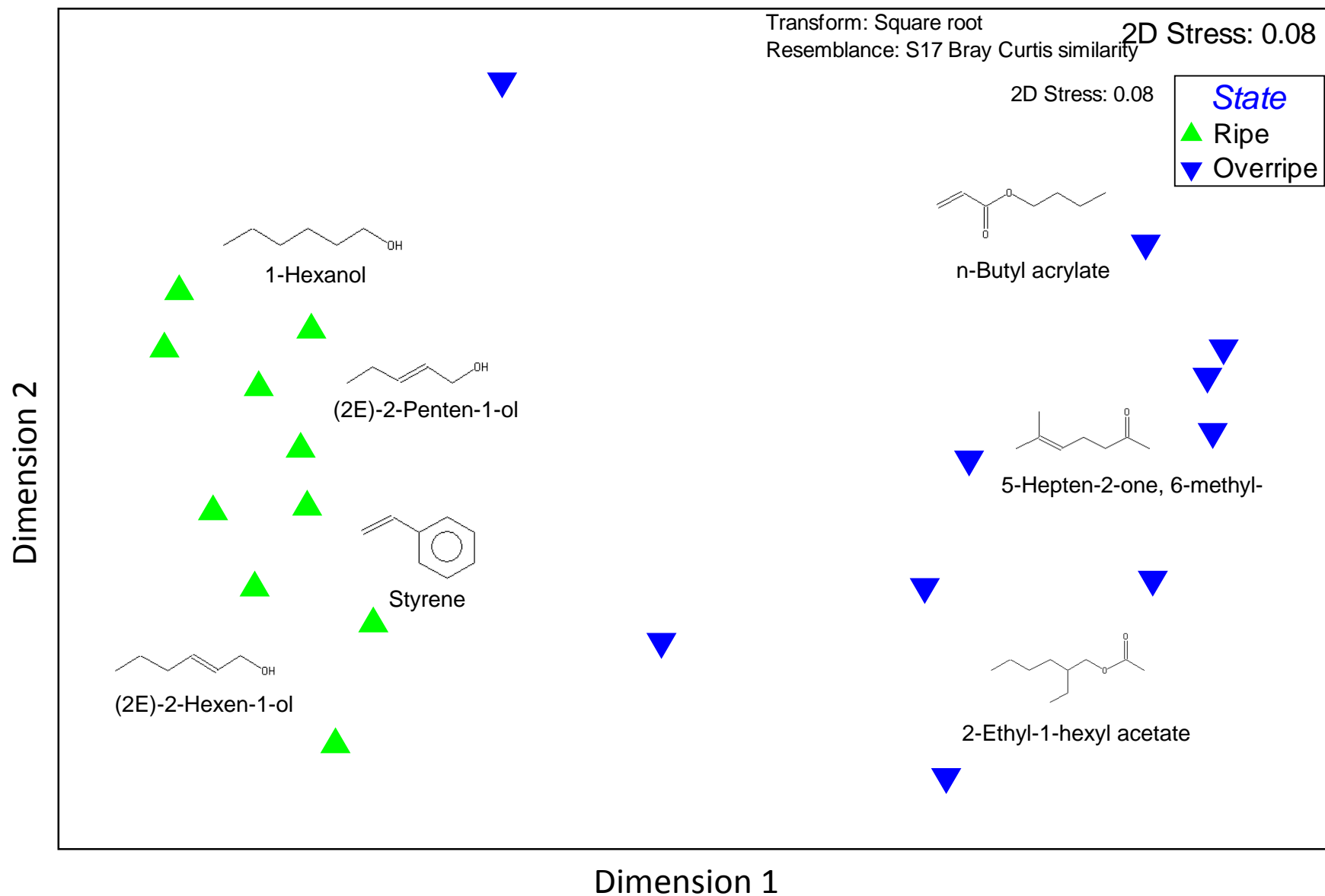


Figure 12. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the variation of compounds emitted during the ripe and overripe stages of *R. globosa*. 2D stress value = 0.08, ANOSIM Global R = 0.871, $P < 0.0001$.

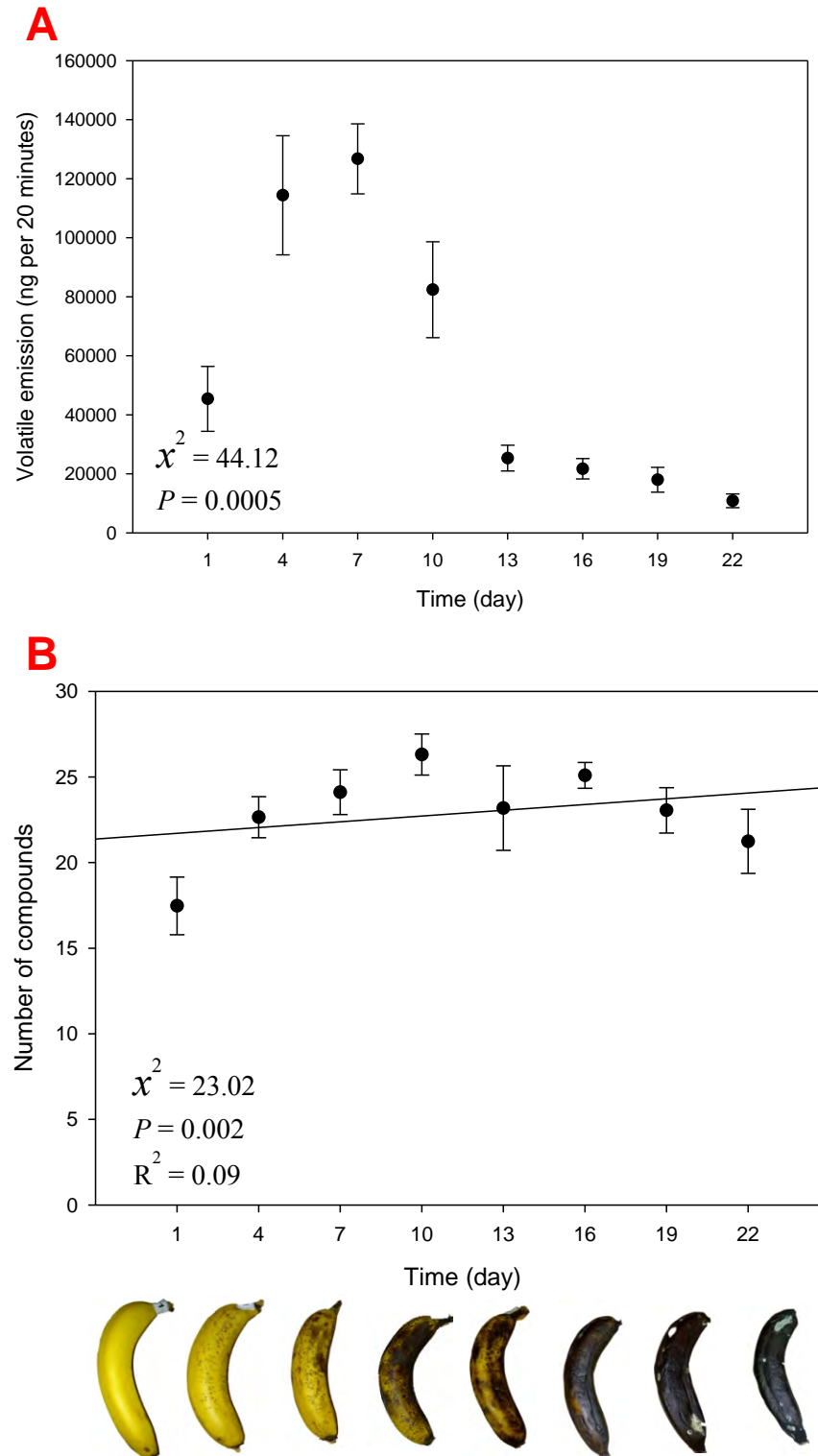


Figure 13. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *M. acuminata*.

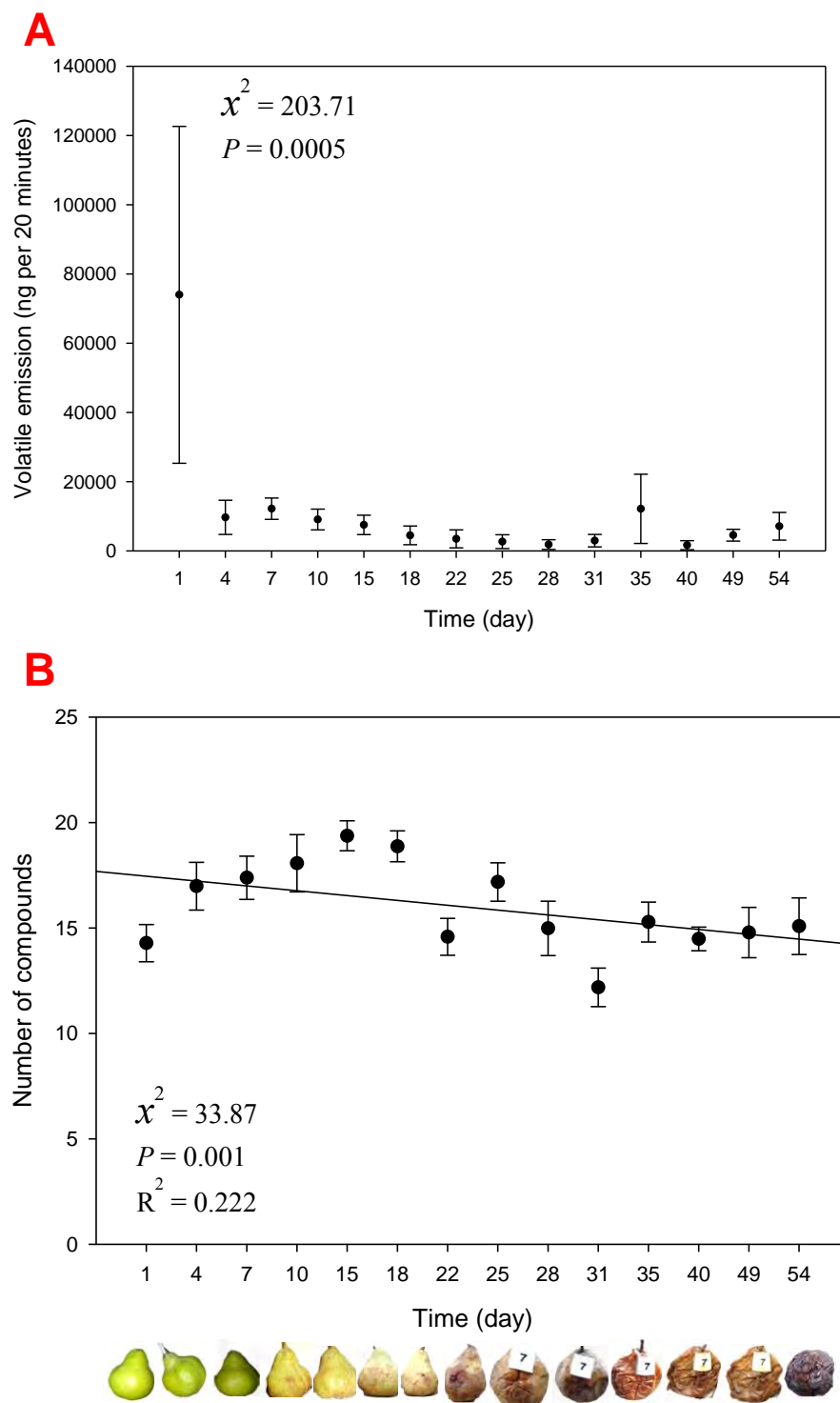


Figure 14. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *P. communis*.

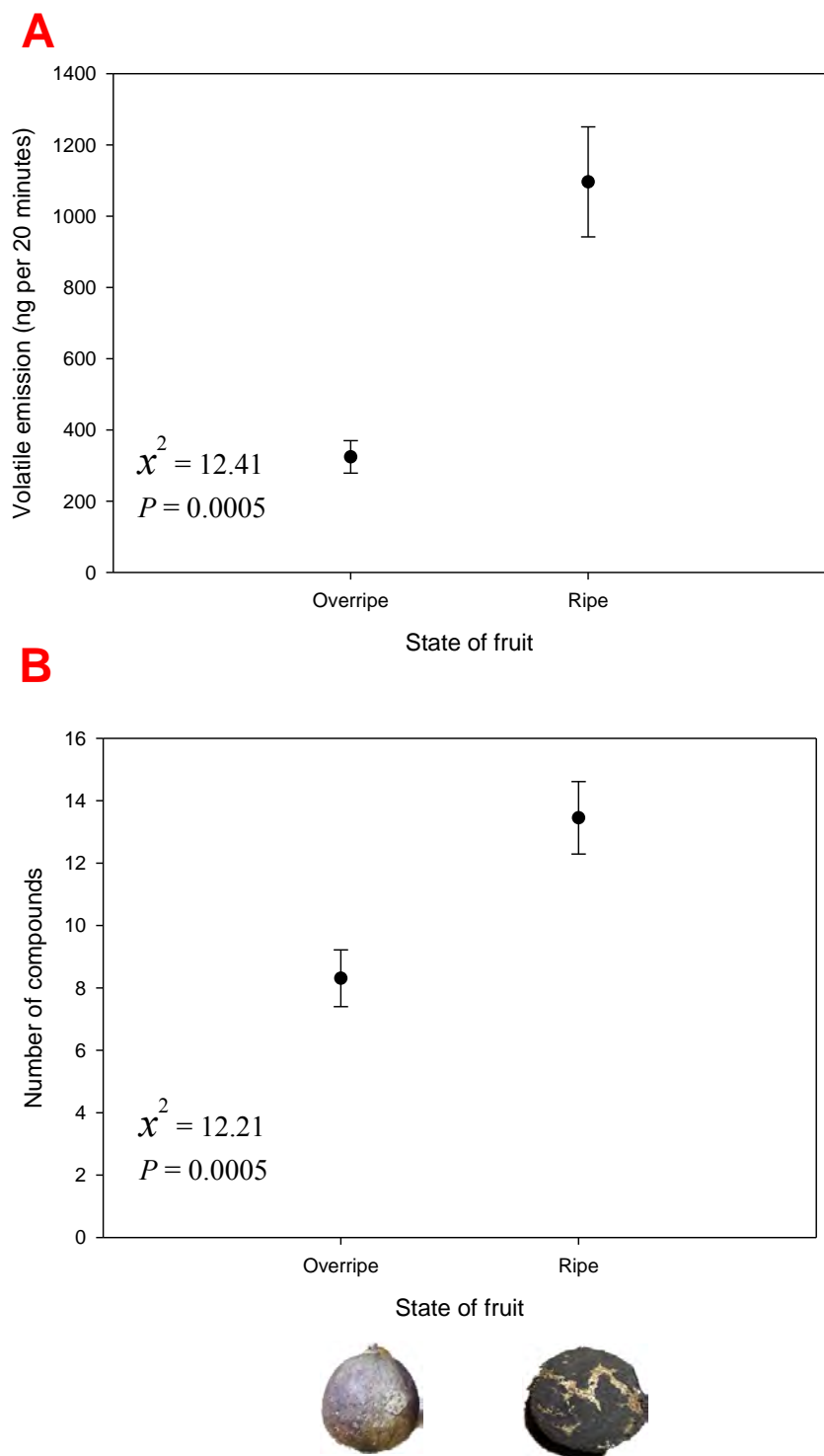


Figure 15. Mean \pm SE of the (A) volatile emission (in ng) of compounds and (B) number of compounds emitted from *R. globosa*.

Table 1: Relative amounts (%) and the standard deviation of compounds identified by GC-MS from headspace samples of *Musa acuminata*, *Pyrus communis* and *Rothmannia globosa*. Scent compounds are listed according to compound class and Kovats retention index (KRI). Compounds only contributing $\geq 1.0\%$ were included.

Compound	RI Kovats	<i>Musa</i> <i>acuminata</i>	<i>Pyrus</i> <i>communis</i>	<i>Rothmannia</i> <i>globosa</i>
Fatty acid derivatives				
Hydrocarbon esters				
Ethyl acetate	950	1.64±2.60	13.08±21.78	—
Isobutyl acetate	1055	2.52±6.28	—	—
Ethyl butanoate	1064	1.26±1.84	—	—
Butyl acetate	1093	—	19.18±18.93	—
3-Methyl-2-butanol acetate	1102	5.50 ± 11.41	—	—
Butyl propionate	1127	—	—	2.11±4.70
Unidentified hydrocarbon ester		—	—	13.32±19.43
Amyl acetate	1159	—	1.75±3.58	—
Butyl acrylate	1189	—	1.80±3.73	—
3-Methyl-1-butanol acetate	1200	12.33±11.55	1.21±2.33	—
Isobutyl butyrate		8.19±7.93	—	—
Butyl butanoate	1206	—	1.69±7.89	—
Amyl isovalerate	1214	4.43±6.68	—	—
Isopentyl 2-methylpropanoate	1216	8.76±14.02	—	—
Ethyl hexanoate	1225	—	1.25±6.11	—
n-Butyl butanoate	1236	1.50±3.55	—	—
Isopentylformate		—	—	3.31±8.47
Hexyl acetate	1240	—	25.62±22.67	1.64±3.75
1-Methylbutyl butyrate		3.47±9.17	—	—
2-Ethyl-1-hexyl acetate		—	1.43±2.66	5.56±8.99
Isoamyl butyrate		19.64±21.63	—	—
2-Methylbutyl 2-methylbutanoate		1.39±5.39	—	—
Isopentyl 3-methylbutanoate		8.55±6.65	—	—

Table 1. Continued.

Compound	RI Kovats	<i>Musa</i> <i>acuminata</i>	<i>Pyrus</i> <i>communis</i>	<i>Rothmannia</i> <i>globosa</i>
<i>Hydrocarbon alcohols</i>				
Isobutyl alcohol	1090	1.81±3.31	—	—
1-Butanol	1115	—	—	4.66±5.56
3-Methyl-1-butanol		11.71±18.18	2.24±4.76	—
1-Hexanol	1347	—	1.87±1.80	29.85±24.12
(3Z)-3-Hexen-1-ol	1358	—	—	1.03±1.25
(2E)-2-Hexen-1-ol	1380	—	—	1.41±1.90
2-Ethyl-1-hexanol	1481	—	9.36±10.38	—
2,3-Butanediol	1592	1.24±3.99	—	—
<i>Hydrocarbon aldehydes</i>				
(E)-2-Hexenal	1212	—	—	3.78±7.13
<i>Hydrocarbon ethers</i>				
Butyl ether	965	—	1.05±2.65	—
<i>Hydrocarbon acids</i>				
Acetic acid		—	6.89±17.47	—
Benzenoids				
Styrene	1265		—	5.85±10.71
Benzyl alcohol	1882		—	6.24±9.85
Phenylethyl Alcohol	1899		1.19±2.85	1.05±1.69
Benzaldehyde	1500		—	10.84±10.25

Chapter 5

Conclusions and future research

In order for plants to persist and increase their reproductive success by means of reproduction, they have developed mechanisms that act as a defense against herbivores and pathogens (Schiestl, 2010). Plants have evolved direct and indirect defense such as visual and olfactory cues. Olfactory cues, which are the result of the release of VOCs from plants, mediate the attraction of pollinators/seed dispersers, and may promote floral constancy and the deterrence of non-beneficial visitors (see Schiestl, 2010). In this study I have explored the emission of late stage floral and fruit VOCs and the changes in the VOC composition during different developmental stages of flowers and fruits.

In chapter one, I explored the idea that microorganisms play an important role for the emission of VOCs from senescing flowers and fruits. VOCs emitted from flowers and fruits serve as an attractant to pollinators and seed dispersers. The primary function of the emission of flower VOCs is to attract pollinators which facilitate the dispersal of pollen which in turn favours the reproductive fitness of the plant (Dudareva et al., 2006). Other functions of the emission of flower VOCs include tri-trophic interactions, plant-plant interactions, belowground interactions and abiotic stresses (Dudareva et al., 2006). The function of the emission of late stage fruit VOCs serve as a deterrent to animals (Sanchez et al., 2004; Sanchez et al., 2006, Sanchez et al., 2009).

In chapter two, I looked at the use of two common extraction techniques that are used for the collection of VOCs, namely: micro solid phase extraction (Micro SPE) and solid phase micro extraction (SPME). It was found that there were differences in the absolute emission when comparing the two methods. Both Micro SPE and SPME have their advantages and limitations. Thus, selecting an adequate extraction technique depends mainly on the specific needs of the user and the type of experiments being carried out. For instance, Micro SPE is better for field experiments because many samples can be taken and then stored in a refrigerator and analysed at a later time. This is not so easy with SPME because relatively expensive field samplers and fibers would make the collection of many samples (e.g. more than 50) expensive and also logistically challenging. An advantage of SPME was the low variability in the absolute amounts

recovered from samples compared to Micro SPE. This study revealed that the high variability of the Micro SPE was the result of bleed-through. One recommendation that follows from the results is that for quantitative analyses the sample filters/traps should be tested for bleed-through before they are used.

In chapter three, the change in the emission of flower VOCs from the time the flower opens until it wilts was investigated. Three plant species were used: *Hymenocallis littoralis*, *Dendrobium chrysotoxum*, and *Camellia reticulata*. Differences in the absolute emission of compounds were found in *H. littoralis* and *D. chrysotoxum*, there was no difference in absolute emission of compounds of *C. reticulata*. Changes in the appearance of the flowers over their life cycle were also visible. The emission of monoterpenes and aldehydes during the fresh stages of flowers could serve to attract pollinators. The emission of these compounds could possibly decrease upon pollination and thereby the emission of esters and alcohols could become a more dominant aspect of the floral bouquet. Such a change in the scent composition could inform flower visitors that the pollination has already occurred and there are no available rewards.

In chapter four the temporal change in the emission of VOCs from the fresh to the rotting stage of three different fruits were investigated. It was found that *Musa acuminata* (banana), *Pyrus communis* (pear), and *Rothmannia globosa* emitted more alcohols and acids during the rotting stages. Changes in the absolute emission of VOCs were found in all three fruit species, with increased emission noted during the rotting stage. The emission of alcohols and esters during the rotting stage of the fruit could be a cue to seed dispersers that the fruit is microbe infested (Dominy, 2004, Levey, 2004). This is supported by the findings of Dominy (2004) and Levey, (2004). These authors reported that rodents and bats are sensitive to fruits infested with microorganisms. A study by Rodriguez et al. (2013), indicated that rodents and bats are sensitive to fruits infested with microorganisms. However there are some specialized rodents that prefer fruits that show signs of decay (see discussion in Rodriguez et al. 2013). Overripe fruits, rich in ethanol would only possibly be consumed rodents and primates, if there is no other available food source (Dominy, 2004).

Overall, temporal variation in the emission of VOCs from fruits and flowers do occur and these likely communicate a message to pollinators and seed dispersers. There are gaps in our knowledge on how these changes may influence the behavior of animals that are important

interaction partners of plants. It would be interesting to investigate in experiments which of the late stage VOCs identified in this study play a role as olfactory cues for animals either also as attractants or repellents. Carrying out tests using blends of floral and fermentation volatiles may enhance our understanding about the potential function of VOCs that are emitted from late stage flowers and fruits. Finally, comparative studies are needed to investigate why some flowers stop emitting VOCs once they have been pollinated, whereas others release a different set of compounds. Are these two different ways to repel pollinators and to make the pollination process more efficient? Similar questions can be asked for the temporal dynamics of fruit- and fermentation VOCs emitted from fruits. A fruitful approach could be to test experimentally the effect of fermentation VOCs on the behavior of fruit dispersers.

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